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UNIVERSITY OF ALBERTA

MOLECULAR AND FUNCTIONAL STUDIES ON NONSENSE SUPPRESSION BY A DROSOPHILA $tRNA^{Ser}_{7}$ GENE

BY

SITA SAILAJA PAPPU

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF GENETICS

EDMONTON, ALBERTA

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Date. Feb. 7, 1990

DEDICATION

Dear mother and Hanumanth,
This thesis is for you both.

ABSTRACT

The anticodon of the wild type $tRNA_7^{Ser}$ gene of *Drosophila melanogaster* was mutated using oligonucleotide-directed, site specific mutagenesis and all three nonsense suppressor derivatives of the gene were constructed. These constructs were tested for functionality in a heterologous system, *Saccharomyces cerevisiae*. For this purpose, all these constructs were sub-cloned into the *Escherichia coli*-yeast shuttle vector, YRp7, and transformed into the *S. cerevisiae* strain JG369-3B(α) that contained an array of tester nonsense alleles. When tested on appropriate omission media, the Drosophila suppressor genes were found to function in *S. cerevisiae* with strict codon specificity. Subsequent Northern hybridization analyses revealed that the Drosophila suppressor genes are transcribed and processed well in *S. cerevisiae*.

The changes introduced into the anticodon of the $tRNA_7^{Ser}$ gene of Drosophila were found to have an effect on the level of transcription and all the mutant constructs were expressed at levels lower than the level at which the wild type Drosophila gene was expressed in *S. cerevisiae*. These results indicated that the wild type anticodon sequence is not indispensable for the transcription and processing of these Drosophila tRNA genes. The varying levels of transcription of these suppressor tRNA genes, which are all biologically active, indicated that expression at the wild type level is not a requirement for efficient suppression.

All the constructs were subcloned into the *E. coli*-Drosophila vector, Carnegie20, and introduced into the Drosophila genome by P element-mediated germline transformations of the Drosophila strain, *b cn;ry*^{8}. Transformants were selected on the basis of *ry*$^{+}$ phenotype and characterized further by Southern hybridization analyses and *in situ* hybridization to salivary gland chromosomes. All the transformants were crossed into the appropriate *Adh*$^{-}$ genetic background containing an appropriate (opal or amber) nonsense mutation. When tested for suppression of the *Adh*$^{-}$ tester nonsense mutation, no

detectable level of suppression was found in the ADH enzyme assays. Southern hybridization analyses of the transformants revealed the presence of the suppressor tRNA genes within the Drosophila genome. *In situ* hybridization and Southern hybridization analyses indicated the presence of one integration event per transformant. Competition assays done using anti-ADH antibodies revealed no detectable increase in the CRM levels in the strains carrying the suppressor tRNA genes. Finally, no detectable levels of suppressor tRNAs were found in the Northern analyses. All these results led to the conclusion that in Drosophila, the failure in demonstrating biological suppression by these suppressor tRNA genes (which are active in *S. cerevisiae*) is due to negligible expression of the suppressor tRNAs in the Drosophila genome. Also, it was speculated that Drosophila might not tolerate suppressor tRNAs above a certain level and therefore, transformants with very low levels of suppressor tRNA expression are the only ones that could be recovered.

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ABBREVIATIONS

Adh: Structural locus for alcohol dehydrogenase,

ADH: The enzyme, alcohol dehydrogenase,

Amp^r: Ampicillin resistance,

box A or 5'ICR: 5' internal control sequence,

box B or 3' ICR: 3' internal control sequence,

bp: Base pair,

BSA: Bovine serum albumin,

CRM: Cross reacting material,

DES: Diethyl sulphate,

EDTA: Ethylene diamine tetra acetic acid,

EMS: Ethyl methane sulphonate,

G_0: Generation-0,

G_1: Generation-1,

I: Inosine,

IAA: Isoamyl alcohol,

kb: Kilo base(s) or 1000 bp,

L: Lysidine,

LTR: Long terminal repeat,

mcmU: Methoxycarbonylmethyluridine,

$m^2{}_2G$: N^2, N^2- Dimethyl guanosine,

mnmU: Methylaminomethyluridine,

NG: N-methyl-N'-nitro-N-nitrosoguanidine,

PAGE: Polyacrylamide gel electrophoresis,

PBS: Phosphate buffered saline (0.01 M sodium phosphate [pH:7.5], 0.14 M NaCl),

Q: Queosine,

R: Purine,

RF: Replicative form,

Spont.: Spontaneous,

SSC (1x): 0.15 M NaCl, 0.015 M sodium citrate,

STE (1x), pH: 7.5: 10 mM Tris.Hcl (pH: 7.5), 100 mM NaCl, 1 mM EDTA,

s^2U: 2-thiouridine,

TAE (1x): 0.04 M Tris acetate, 0.001M EDTA,

TBE (1x): 0.089 M Tris borate, 0.089 M boric acid, 0.002M EDTA,

TE (1x), pH: 8.0: 10 mM Tris.Hcl (pH: 8.0), 1 mM EDTA,

TEMED: N, N, N', N'- Tetramethylethylenediamine,

TGE (1x): 50 mM glucose, 25 mM Tris.Hcl (pH: 8.0), 10 mM EDTA,

TMV: Tobacco mosaic virus,

ψ: Pseudouridine,

V: Uridin-5-oxyacetic acid,

Xdh: Structural locus for xanthine dehydrogenase,

XDH: The enzyme, xanthine dehydrogenase,

Y: Pyrimidine,

Wye base: α-(carboxyamino)-4,9-dihydro-4,6-dimethyl-9-oxo-1H-imidazo[1,2-a]-purine-7-butyric acid dimethyl ester

Chapter I

INTRODUCTION

Suppression is defined as the restoration of a wild type or pseudo-wild type phenotype in a mutant due to a second mutation at a site distinct from that giving rise to the initial mutant phenotype (reviewed in Eggertsson and Adelberg,1965; Steege and Söll, 1979; Kubli, 1986). This phenomenon of suppression can be brought about by various mechanisms, and can be due to interaction of either allelic (intragenic suppression) or non-allelic (intergenic suppression) mutant genes (reviewed in Kubli, 1986).

The concept of tRNA mediated suppression originated with Benzer's work on the rII region of the bacteriophage T4 (Benzer and Champe, 1962). When some of the rII mutants were grown on certain bacterial hosts, the mutant phenotype disappeared. These differences were considered to be due to a kind of heritable suppressor mutation in those bacterial hosts. The work of Crick and co-workers on acridine-induced rII mutants reinforced this concept (Crick et al., 1961), because the effect of one class of mutations (+ frame shift mutation) could be reversed by mutations of the opposite (-) type within the same cistron. The latter example falls under the category of intragenic suppression. The second type, intergenic suppression, can be accomplished by various mechanisms, and a particularly important class is that of informational suppressors which alter the fidelity of translation of mutant messages to allow the production of full length gene product, by changes in either transfer RNA (Eggertsson and Adelberg,1965; Garen,1968; Goodman et al,1968; and for more recent reviews see later), or some other component (eg., ribosomal protein) of the translational machinery (Gorini,1970).

Since the focus of this research is tRNA-mediated nonsense suppression, the structural properties of the tRNA molecule and its functional aspects are briefly reviewed. This is followed by a summary of tRNA mediated nonsense suppression in both

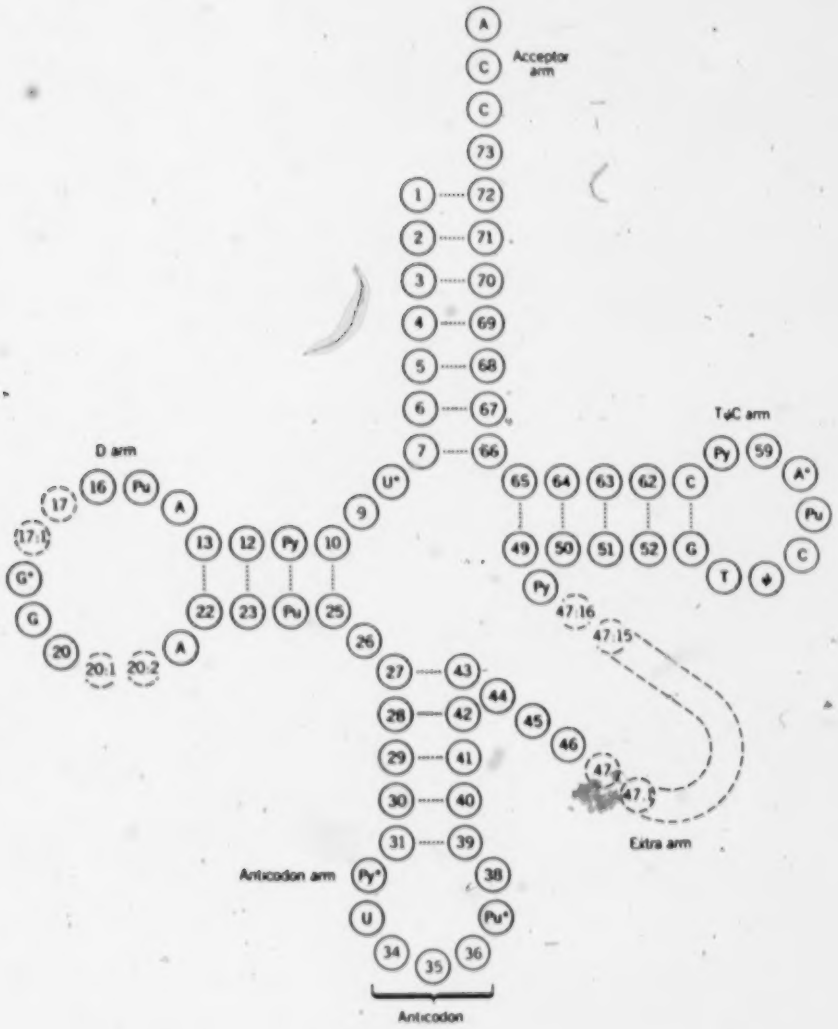
prokaryotes and eukaryotes. Finally, there is a synopsis of the phenomenon of suppression and the search for nonsense suppressors in *Drosophila melanogaster*.

Transfer RNAs are small RNA molecules within the cells, which are involved in the transfer of amino acids to the growing polypeptide chain during translation. These molecules play a key role in decoding the nucleic acid sequence of the messenger RNA into a sequence of amino acids in proteins. This is facilitated by the covalent attachment of one specific amino acid to their 3' end, and recognition of the corresponding 'cognate' mRNA codon of that amino acid, by their anticodon sequence. Thus, they play the role of the 'adaptor' molecule, first proposed by Crick in 1955 (described in Zachau, 1978).

The primary structure or sequence of many prokaryotic and eukaryotic tRNAs and their genes has already been determined (Sprinzl et al., 1989). The tRNA molecules are a mixture of species ranging from 73-92 nucleotides in length and they sediment at about 4S in the analytical ultracentrifuge. The sequences of the tRNAs vary widely between and within species, and are characterized by a relatively large molar proportion of modified or unusual nucleosides within their sequences. In spite of this variation, some of the nucleosides within tRNA sequences are considered invariant. These include, U_8, A_{14}, G_{18}, G_{19}, A_{21}, U_{33}, G_{53}, T_{54}, ψ_{55}, C_{56}, A_{58}, C_{61}, C_{74}, C_{75}, and A_{76} (Fig. 1). Some of the other positions in the tRNA are 'semi-invariant'. That is, those positions are always occupied by either a pyrimidine (Y) or a purine (R). Such positions include: Y_{11}, R_{15}, R_{24}, Y_{32}, R_{37}, Y_{48}, R_{57}, and Y_{60} (Sprinzl et al., 1989). The 5' nucleotide of the tRNA always carries a 5' terminal phosphate group and is often a G. The 3' end always terminates in a CCA-OH sequence, which is encoded in the gene in the case of some prokaryotes, and is added post-transcriptionally in the case of eukaryotes.

There are some exceptions to the rules mentioned above. The initiator tRNAs of both prokaryotes and eukaryotes fall into this category. Prokaryotic $tRNA_f^{Met}$ lacks a base pair in the acceptor stem, and the Y_{11}-R_{24} base pair in the D-stem is replaced by an A_{11}-U_{24} base pair. Similarly, in the case of eukaryotic initiator tRNA, an A_{54} and U_{55} replace

Fig. 1 The universal cloverleaf structure of a tRNA molecule. Circles indicate base positions, the invariant bases are indicated by the particular base present at that position. For semi-invarinat bases, Py and Pu indicate the presence of a pyrimidine or a purine. An asterisk indicates that the base is modified but that the form of the modificaiton may vary. Dots indicate base pairing. The anticodon position is shown with a bracket (modified from Kim et al., 1974 and Lewin, 1987). Bases shown with broken circles represent those bases that may not be present in all the tRNA molecules.


A
C
C
73
1
72
2
71
3
70
4
69
5
68
6
67
7
66
Acceptor arm
D arm
TψC arm
16
Pu
17
17:1
A
13
12
Py
10
9
U*
G*
G
22
23
Pu
25
20
20:1
20:2
A
26
65
64
63
62
C
49
50
51
52
G
Py
59
A*
Pu
C
T
ψ
Py
47:16
47:15
27
43
44
28
42
45
46
29
41
47:1
30
40
47:6
31
39
Extra arm
Anticodon arm
Py*
38
U
Pu*
34
35
36
Anticodon

the invariant T_{54} and ψ_{55}. In addition, in higher eukaryotes, the $tRNA_i^{Met}$ has a C residue at position 33 (instead of the invariant U_{33}), adjacent to the anticodon (Addison, 1982; Sprinzl et al., 1989). The deviations of the initiator tRNAs from the generalized structural rules might reflect their special role in the translation of the message. Also, organellar tRNAs are probably exceptional since they are smaller.

Over 50 modified bases have been identified from tRNAs of different organisms. Many of these modified bases differ from normal bases by enzymatic modifications of the pre-existing bases, methylation of ribose moieties, or replacement of the oxygen atoms in the bases by sulphur (Kim, 1978; reviewed in Nishimura, 1978; Bjork, 1984). The function of these modified nucleosides is not completely understood, but the occurrence of these bases at some characteristic positions in the tRNA seems to suggest that they play a significant role in tRNA structure and function. All known tRNA modifications, except two, occur at the polynucleotide level; that is, they involve alteration of, or addition to, the existing bases. One of the exceptions involves the formation of the Q base which occurs at the monomeric level (by tRNA transglycosylase) where the modification enzyme exchanges free queosine with a guanine residue in the tRNA. A similar reaction results in the insertion of inosine (I) at the wobble position of some anticodons (reviewed in Bjork, 1984).

The presence of modified nucleosides at position 37 (3' to the anticodon) correlates with coding specificity. For example, tRNAs reading UXX (for example, serine) codons have a bulky hydrophobic residue like N6-isopentenyl adenosine (i^6A), or some derivative of the Wye base at position 37, and those reading AXX (for example, lysine) codons have a hydrophilic residue like N-[(9-β-D-Ribofuranosylpurin-6-yl)carbamoyl]threonine (t^6A) at position 37. These residues are thought to strengthen the weak A-U base pairing at the 3' position of the anticodon with the 5' position of the codon. G or C residues at the third position of the anticodon are followed by simple methylated purines or by unmodified A.

Modified nucleosides are also often found at the 'wobble' position or position 34 of the anticodon. The concept of wobble base pairing describes the ability of a tRNA to

recognize more than one codon by unusual (non-G-C, A-U) pairing of the first (5' end) base of the anticodon with the third (3' end) base of a codon. For example, the base U, when present at the wobble position is capable of recognizing codons ending with A (by standard base pairing) or with G (by wobble base pairing). All tRNAs from eubacteria and eukaryotes, that read codons ending with U or C, have Q (queosine) or a derivative of it at position 34. Also, an unmodified A or U is almost never found at the anticodon wobble position. An A at position 34 may be modified to I (inosine) which can efficiently pair with A, U, or C in the 3' position of the codon; and 'U' at this position of the anticodon may be modified to s^2U in tRNAs that read codons ending with A or G. These modifications are thought to increase the wobble capacity of the anticodon. A Q-U wobble pair is supposedly three times stronger than the G-U wobble pair. Some modified bases like 'mcmU' or 'mnmU' are thought to restrict the wobble base pairing. The content of Q base in Drosophila tRNA changes during development (White et al.,1973), and Q is completely lacking in tumor cells (Okada et al.,1978). These observations might reflect that some modified bases have a translational regulatory role in different systems and cell types.

The function of the modified bases in places other than the anticodon region is even less clear. The presence of ψ in the anticodon stem, in the case of $tRNA^{His}$, was shown to influence translational fidelity and efficiency in *Salmonella typhimurium* (Parker.,1982; Palmer et al.,1983). In yeast, deficiency of m^2_2G at position 26 of $tRNA^{Ser}$ results in a reduction in growth rate and charging capacity. This modification is thought to stabilize the three dimensional structure of the tRNA molecule, or perhaps the modified bases participate in specific interactions with different macromolecules. The exact nature of the role played by these modified bases in the structure and function of the tRNA is still elusive (see Bjork, 1984 and references cited therein for more details).

In general, it is thought that the main role of base modifications in tRNA is in the modulation of translational efficiency and codon specificity (reviewed in RajBhandary, 1988). Base modifications in and around the anticodon sequence of the tRNA increase

translational efficiency of the tRNA and fidelity of the translation process. However, recent findings of Muramatsu et al. (1988) suggest another interesting constraint conferred on the tRNA by base modification to this region. In their work, it was shown that a single post-transcriptional modification changed the codon and amino acid specificities of an *E. coli* tRNA. *E. coli* has two $tRNA^{Ile}$ species, a major species with a GAU anticodon (reading AUU and AUC codons), and a minor species with a LAU anticodon (reading AUA). L in the anticodon stands for lysidine, a modified base derived from cytosine in which the 2-keto group is replaced by the amino acid lysine. Lysidine is thought to pair with only A in the codon. Muramatsu et al. (1988) demonstrated that changing the lysidine to cytosine in the anticodon made this $tRNA^{Ile}$ (now with a CAU anticodon) a very good substrate for methionyl tRNA synthetase, with a corresponding change in the coding specificity of this tRNA. It was postulated that this could be a simple mechanism for *E. coli* to minimize translational errors arising from partial modification.

Secondary structure of tRNA: The universal cloverleaf

Despite the variation in the primary sequence/structure of the tRNAs, all of them can be drawn into a cloverleaf secondary structure. The common features of this structure (Fig. 1) include: a) an acceptor stem containing the 5' and 3' ends of the molecule. It contains an unpaired sequence of four bases (GCCA) at the 3' end and it is to the 2' or 3' free -OH group of the A in this sequence that the amino acid is covalently esterified; b) a D-stem and loop which derives its name from the presence of dihydrouridine in the loop structure; c) an anticodon stem and loop; and, d) a TψC stem and loop containing those three bases in the loop structure.

The stem structures usually show Watson-Crick base pairing but occasionally unusual base pairs (example, G-U, G-ψ, A-ψ) are seen. The number of bases and base pairs in the loops and stems of the secondary structure are fairly constant. The aminoacyl acceptor stem contains seven base pairs, the D-stem usually three and sometimes four base

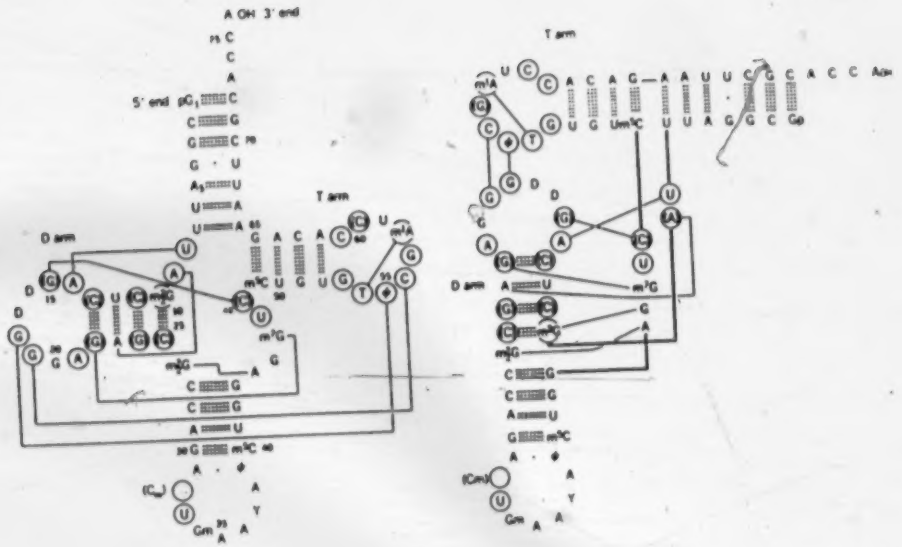
pairs; the D-loop eight to eleven nucleotides; the anticodon and TψC stems contain five base pairs each and their loop structures seven nucleotides each. In addition to these, an extra arm (present between the anticodon arm and the TψC arm) is present in all the tRNAs. The length of this arm is extremely variable, and the tRNAs can be grouped into two classes based on its length. Class I tRNAs contain a small extra arm of about 3-5 bases, and most of the tRNAs fall into this category. Class II tRNAs have an extra arm consisting of 13-21 nucleotides with about 5 base pairs in the stem. The $tRNA^{Leu}$, $tRNA^{Ser}$ and prokaryotic $tRNA^{Tyr}$ belong to this category. Organellar tRNAs, however, are smaller (59-75) than their cytoplasmic counterparts and often do not conform to the generalized structure described above. For example, a 59 nucleotide $tRNA^{Ser}$ from mammalian mitochondria has lost the D stem and loop structure (Arcari and Brownlee, 1980; de Bruijn et al.,1980).

The tertiary structure of the tRNA

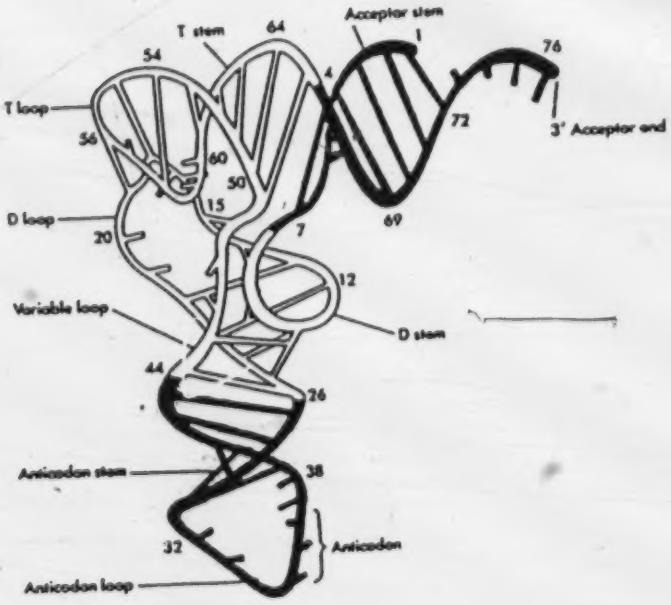
X-ray crystallographic studies revealed that the tRNA can fold into a higher order structure (reviewed in Kim, 1978) usually described as L-shaped. The crystal structure of the yeast $tRNA^{Phe}$ was the first to be elucidated (Rich and RajBhandary, 1976). The structure is 2.0-2.5 nm thick (Fig. 2), and consists of two major arms or domains. The first domain consists of the acceptor stem and TψC stem/loop, with the terminal CCA sequence located at its extremity. The other domain consists of the variable D-stem/loop and the anticodon stem/loop structures, with the anticodon loop at the molecule's other extremity. The TψC and D-loop hydrogen bond at the corner of the molecule. The dimensions of the two domains are very similar; about 6.0 nm from the corner to either end of the molecule and about 7.5 nm between the extreme ends.

The stability of the tertiary structure is brought about by extensive base stacking and unusual hydrogen bonding between bases that are not adjacent in the cloverleaf structure. Many of the tertiary interactions are between the invariant or semi-invariant bases found in

Fig. 2 Tertiary structure of the yeast phenylalanine tRNA. Fig. 2A and 2B represent the tertiary interactions of hydrogen bonding and base stacking seen in the tRNA molecule. The unbroken lines represent the bonds joining the arms in the L-shaped molecule. Fig. 2C is a diagrammatic illustration of the yeast $tRNA^{Phe}$ molecule. The numbering of the nucleotides is the same as in Fig. 1 (adapted from Kim et al., 1974).


A OH 3' end
5' end
D arm
T arm
Acc
(Cm)
Gm

(A) (B)


T stem
Acceptor stem
T loop
D loop
Variable loop
D stem
Anticodon stem
Anticodon
Anticodon loop
3' Acceptor end

(C)

the tRNA molecule (see Kim, 1978 for details), which lends support to the idea that the tertiary structure deduced for $tRNA^{Phe}$ of yeast is representative of the three dimensional structure for all the tRNAs. The tertiary structures of some other tRNAs have also been reported. These include those of yeast $tRNA^{Asp}$ (Moras et al., 1980), yeast $tRNA^{Met}$ (Shevitz et al., 1980), and *E. coli* $tRNA^{Met}$ (Woo et al., 1980). The general base stacking and hydrogen bonding interactions found in these latter cases conformed to that of the yeast $tRNA^{Phe}$, suggesting that this type of tertiary structure is indeed typical of all tRNAs. The inner surface of the two domains of this L-shaped molecule contains most of the variable bases of the D-stem and variable loop. These bases are not involved in any of the tertiary interactions stabilizing the structure. They may play a role in recognition of the specific tRNA molecule by its cognate aminoacyl tRNA synthetase.

Number and organization of the tRNA genes

In *E. coli* 54 tRNA genes, encoding 35 sub-species, and representing an estimated two-thirds of the total tRNA genes, have been mapped (reviewed in Fournier and Ozeki, 1985). The chromosomal map of *E. coli* shows that these tRNA genes are dispersed throughout the genome. Of the 54 tRNA genes, 37 occur in polycistronic operons, of which 14 are in the seven rRNA operons. The remaining occur in six operons that encode three to seven tRNAs per cluster. All the *E. coli* genes encode the 3' -CCA terminus of the tRNA.

While prokaryotes contain one to a few copies of the genes encoding a particular tRNA species, eukaryotic tRNAs are usually encoded by multicopy genes. The organization of these genes varies from species to species (reviewed in Sharp et al., 1985). In yeast, there are about 360 tRNA genes which are dispersed throughout the genome. In Drosophila, however, the estimates of gene number vary. The total number was estimated to be about 750 per haploid genome by Ritossa et al. (1966) and Tartof and Perry (1970). A lower estimate of 590 copies per haploid genome was reported by Weber and Berger

(1976). The genes were found to be arranged in approximately 60 clusters scattered over most of the chromosomes. Within a cluster, the arrangement is totally irregular and each cluster might contain several types of tRNA genes, some in multiple copies. Furthermore, they are irregularly spaced and transcribed in both directions (see Sharp et al., 1985). In the human genome, there are about 1000 tRNA genes composed of about 60 different sequences (Hatlen and Attardi, 1971). Evidence suggests that the organization of the tRNA genes is very similar to that found in Drosophila, in that there are also clusters of different tRNA genes scattered all over the genome (Santos and Zasloff., 1981; Roy et al., 1982; Goddard et al., 1983; MacPherson and Roy., 1986; Doran et al., 1988).

About 10-20% of nuclear encoded tRNA genes in eukaryotes contain introns (reviewed in Sharp et al., 1985). These intervening sequences range from 8 to 60 nucleotides in length and are homologous within any isoacceptor family, but among different gene families the introns are completely divergent. Introns appear to be rare in Drosophila, and until now, only a pair of tightly linked $tRNA^{Leu}$ genes (Robinson and Davidson, 1981) and $tRNA^{Tyr}$ genes (Suter and Kubli, 1988) are reported to contain introns. The intron of one of the $tRNA^{Tyr}$ genes in Drosophila is 113 bp in length and is the longest of the known cytoplasmic tRNA gene introns in Drosophila (Suter,1987). The maize chloroplast isoleucine and alanine tRNA genes contain intervening sequences of 949 bp and 806 bp respectively and are the longest of all known tRNA genes (Koch et al., 1981). Whenever present, the location of the introns is always one base pair 3' to the anticodon in all the tRNA genes examined (Abelson, 1979; Sprinzl et al.,1989).

Transcription of the tRNA genes

The promoters for eukaryotic genes transcribed by RNA polymerase III do not lie in their 5' flanking sequences, but within the coding region of the gene itself (see Korn, 1982; Hall et al., 1982; Brown, 1984 for reviews). Deletion studies on Xenopus 5S RNA genes (Sakonja et al., 1980; Bogenhagen et al., 1980) led to this surprising finding, and

similar studies done on tRNA (Hall et al., 1982) genes revealed that they also have internal promoters, but split into two regions. These two regions were referred to as box A or the 5' ICR (Internal Control Region) and box B or the 3' ICR. Box A extends from nucleotides 8 to 19 and box B from nucleotides 52-62. The distance between the A and B boxes can vary from 31 to 74 bases because of the variable loop and the possibility of introns. Comparisons of the sequences of tRNAs and tRNA genes (Sprinzl et al., 1989) have led to the identification of consensus sequences for these promoter elements (reviewed in Sharp et al., 1985). The sequence corresponding to box A is 5'-TRRYNNARYGG-3' within the D-arm, and that corresponding to box B is 5'-GGTTCGANTCC-3' found within the TψC arm. The sequence of box B is highly conserved, while that of box A is more variable. The conservation found in B box sequence may indicate its importance for the proper function of the tRNA itself, or it may reflect a more conserved sequence requirement for interaction with the transcription factor (TF IIIC) that is thought to bind to the box B. Although the box A sequence is variable between organisms, within any particular species a specific box A sequence is required for proper transcription of the tRNA genes. Mutational studies done with a yeast $tRNA^{Leu}$ gene (Mattoccia et al., 1983; Baldi et al., 1983; Newman et al., 1983), using synthetic oligonucleotides support this conclusion. Base changes introduced into the box A sequence in the D stem region decreased the transcription levels in the homologous system even when the D stem/loop structure was maintained. However, in a heterologous system (Xenopus, in this case), the box A conserved sequence was found not to be critical for proper transcription of this specific yeast tRNA gene. Therefore, the variability found in box A sequences may be conferring a sequence context, specific for homologous transcription factors in any particular species.

Studies done with purified RNA polymerase-III (Parker et al., 1976), with the Xenopus oocyte transcription system, and a partially purified RNA polymerase-III transcriptional complex from Drosophila Kc cells (Burke et al., 1983) suggest that other

factors are required for the transcription of the tRNA genes in addition to RNA polymerase-III. Fractionation of cytoplasmic extracts from human KB cells (Segall et al., 1983) identified two distinct fractions that are required for transcription. Box B of tRNA genes was shown to stably bind a protein present in one of the fractions (Lasser et al., 1983; Newman et al., 1983). This protein was later identified as TF-IIIC and has been partially purified from yeast (Ruet et al., 1984) and HeLa cells (Fuhrman et al., 1984). Other studies (see Lasser et al., 1983 for a review) identified another transcription factor, TF-IIIB, involved in tRNA gene transcription. A general mechanism of tRNA promoter recognition was proposed by Dingermann et al. (1983). According to their hypothesis, the transcription factor IIIB binds to box A and IIIC to box B. The 5' and 3' flanking sequences are required for proper and stable complex formation.

Comparison of the flanking sequences of tRNA genes revealed high conservation of the 5' and 3' flanking sequences among members of at least a few tRNA gene families (reviewed in Sharp et al., 1985). These include: the genes for $tRNA^{Met}$ (Sharp et al., 1981a), $tRNA^{Glu}$ (Hosbach et al., 1980), $tRNA^{Gly}$ (Hershey and Davidson, 1980) and $tRNA^{Arg}$ (Newton et al., 1987) of *D. melanogaster;* two human $tRNA_i^{Met}$ genes (Santos and Zasloff, 1981), two human $tRNA^{Asn}$ genes (Ma et al., 1984); three rat $tRNA^{Asp}$ genes (Shibuya et al., 1982); and the $tRNA^{Trp}$ genes of *D. discoideum* (Peffley and Sogin, 1981). The sequence homology was often found to extend to several hundred base pairs. It was suggested that this could be a reflection of either unequal crossing over events ($tRNA^{Arg}$, Newton et al., 1987; $tRNA^{Glu}$, Hosbach et al., 1980; of Drosophila) or transposition like mechanisms ($tRNA^{Trp}$ of Dictyostelium, Peffley and Sogin, 1981).

Sequences having both positive and negative modulatory effects on the expression of the tRNA genes have also been reported in both the 5'- and 3'- flanking sequences. Negatively acting 5' modulatory sequences, resembling termination signals for RNA polymerase-III, have been reported to inhibit the *in vitro* transcription of $tRNA^{Lys}$ (De Franco et al., 1980; De Franco et al., 1981) and $tRNA_2^{Arg}$ (Dingerman et al., 1982) genes

of Drosophila. The position of these signals varied for different genes, and also the effect was found to be species-specific. For instance, the $tRNA_2^{Ala}$ gene of *Bombyx mori* has two blocks of five T residues within the first 21 bp 5' to the gene. Yet this tRNA gene is found to be constitutively expressed in the silkworm, as well as efficiently transcribed *in vitro* (Sprague et al., 1980).

Positive modulatory effects of 5' flanking sequences have also been reported: in the case of the $tRNA_2^{Arg}$ (Sharp et al., 1981b), and $tRNA_4^{Val}$ (Sajjadi and Spiegelman, 1989) genes of Drosophila ; the $tRNA_3^{Leu}$ gene of *S. cerevisiae* (Raymond and Johnson, 1983; Frischloff et al., 1984; Raymond et al., 1985); the human $tRNA^{Glu}$ gene (Goddard et al., 1983), and the $tRNA_2^{Ala}$ (Larson et al., 1983; Young et al., 1986) gene of *Bombyx mori*. Separate and distinct 5' flanking sequences stimulating transcriptional activity and factor binding have been reported more recently for a mouse $tRNA^{Asp}$ gene (Rooney and Harding, 1988). Further, transcription start site selection was found to be modulated by a discrete region 22 base pairs upstream of the initiation site for a $tRNA^{Asn}$ gene of Drosophila (Lofquist et al., 1988). The varying positions of all these elements might be reflecting a specific context requirement of the different tRNA genes for efficient transcription. Examples of the influence of 3' flanking sequences on the transcription of the tRNA genes include the $tRNA^{Ser}$ gene (St. Louis and Spiegelman., 1985) of Drosophila and the $tRNA_2^{Ala}$ gene of *Bombyx mori* (Wilson et al., 1985), where specific 3' flanking sequences were found to have a positive modulatory effect on transcription.

A recent report by Horvath and Spiegelman (1988) indicated that sequences between the ICk's of the $tRNA^{Arg}$ gene of Drosophila might have a stimulatory influence on the transcription of a $tRNA^{Val}$ gene found downstream of the $tRNA^{Arg}$ gene. They showed that the sequence between the +22 and +36 of the $tRNA^{Arg}$ gene is required for maximum *in vitro* transcription of the $tRNA_{3b}^{Val}$ gene.

Maturation and processing of the tRNA

Eukaryotic tRNA genes are transcribed into precursor molecules, which are larger than the mature sized tRNA (see Hopper et al., 1981; Hopper, 1984; Deutscher, 1984; Sharp et al., 1985 for reviews). These precursor transcripts are formed into the mature sized tRNA within the nucleus of the cell by a variety of processing and modification enzymes. Transcription initiates within ten nucleotides upstream of the first nucleotide of the mature tRNA and normally starts with a purine residue. The transcripts terminate in a stretch of T residues (usually 4-5) located just distal to the encoded 3' end of the mature tRNA sequences. Precursors are subsequently cleaved into mature transcripts by removal of the additional nucleotides at the 5' and 3' ends, and splicing out of the intron sequence if present. This is followed by addition of the CCA sequence at the 3' end and completion of base modifications. A certain level of modificatipn presumably occurs before the excision of the intron since a proper conformation of the molecule maintained by certain unusual base pairs involving modified bases, is required for efficient excision of the intron (Baldi et al., 1983; Ogden et al., 1984; Swerdlow and Guthrie, 1984; Lee and Knapp, 1985; Mattoccia et al., 1988; Reyes and Abelson, 1988).

The processing of the 5' end requires RNase P, an endonuclease, first identified in *E. coli* (Shimura et al., 1980) by both genetic and biochemical means. An enzyme with a similar activity, that can process the 5' end of a variety of yeast and *E. coli* precursor tRNAs, has been partially purified from *S. pombe* (Kline et al., 1981). Studies done with yeast precursor $tRNA^{Tyr}$ in Xenopus oocytes (Melton et al., 1980; Hopper and Kurjan, 1981) revealed that specific processing of the 5' and 3' ends, and of the intervening sequence, only occurs in the nucleus. They also suggested, that in Xenopus oocytes, the 5' leader is removed in three stages. Therefore, the 5' end removal might not be occurring in a single catalytic step, as was suggested by the initial *in vitro* studies (Shimura et al., 1980; Kline et al., 1981).

The order in which all these events occur is not quite clear. Initially it was suggested (Peebles et al., 1983; Greer et al., 1983) that the processing probably occurs in the following order: endonucleolytic removal of 5' leader and 3' trailer, addition of the 3' terminal CCA, site specific base modifications, and when present, excision of the intervening sequence. According to Leontis et al. (1988), however, the removal of the intron probably precedes the addition of the CCA sequence. These two latter steps could, actually, be occurring in a very short period of time and, thus, might overlap with each other in time.

Aminoacylation and identity of the tRNA

The accuracy of translation depends on two successive and independent steps, viz., matching an amino acid with its cognate tRNA and secondly, matching these charged tRNAs with their codons in the mRNA (reviewed in de Duve, 1988). The specificity of latter step is achieved by the interaction between the codon and the anticodon within the ribosome. The former, however, is more complex. Each amino acid is usually represented by more than one tRNA in the cell. Isoaccepting tRNAs are a group of tRNAs that have the same amino acid specificity and are charged with the cognate amino acid by a single aminoacyl tRNA synthetase. Therefore, each group of isoaccepting tRNAs must share some conserved features which enable the synthetase to distinguish one isoacceptor group from another. Earlier studies, done with emphasis on the structure and sequence of the synthetases (reviewed in Schimmel, 1987), did not shed much light on this matter. More recent studies of tRNA structure/function relationships have begun to reveal those features of tRNA isoacceptors recongized by the synthetase (see de Duve, 1988; Yarus, 1988; Schulman and Abelson, 1988 for reviews).

Studies on $tRNA^{Ala}$ of *E. coli* (Hou and Schimmel, 1988) revealed that as little as a single base pair can determine the amino acid specificity of a tRNA. By site specific mutational studies, they have demonstrated that substitution of the G-U base pair in the

acceptor helix with G-C or A-U base pairs eliminated the aminoacylation with alanine *in vitro* and *in vivo*. Introduction of this base pair into the analogous position of a $tRNA^{Cys}$ and a $tRNA^{Phe}$ conferred upon each, the ability to be efficiently aminoacylated with alanine. Previously, Normanly et al. (1986b) showed that a $tRNA^{Leu}$ of *E. coli* could be transformed into a $tRNA^{Ser}$ by changing 12 nucleotides, but it was not established in their work which replacements were critical.

These findings led to the coining of the term "paracodon" to refer to this 'second genetic code' that is imprinted into the structural features of the tRNAs and the aminoacyl tRNA synthetases, enabling the latter to match the amino acids with the structure of the tRNAs (de Duve, 1988). Later on, this term was replaced by a better term, "tRNA identity", which describes the features of a tRNA molecule which make that tRNA recognizable to one synthetase only.

Other studies, similar to those mentioned above, were done using two different methods. Firstly, *in vitro* transcripts were synthesized by T7 DNA polymerase, using as templates tRNA genes driven by a bacteriophage T7 late promoter (Sampson and Uhlenbeck, 1988). The specificity of charging was monitored by determination of the kinetic parameters of acylation of the tRNA by cognate and noncognate amino acids. In the second method, the alteration of amber suppressor specificity was used as a way of assaying the changes in specificity. The suppressor genes were synthesized *in vitro* (Normanly et al., 1986b) and specificity was assayed by suppressing the amber mutation in the dihydrofolate reductase gene. The amino acid inserted into the protein was determined by protein sequencing.

In this manner, the nucleotides comprising the "identity set" were determined for several tRNAs (see the reviews mentioned above). These include $tRNA^{Met}$ and $tRNA^{Val}$ (Schulman and Pelka, 1988), $tRNA^{Gln}$ (Yaniv et al., 1974), and $tRNA^{Phe}$ (Sampson et al., 1989; McClain and Foss, 1988a). In these cases, in addition to other bases in the tRNAs, the anticodon sequence was found to be an essential identity determinant for proper

aminoacylation. The other examples, where the anticodon is not essential, include $tRNA^{Ser}$ (Schulman and Abelson, 1988), and $tRNA^{Ala}$ (Hou and Schimmel, 1988; McClain and Foss, 1988b). In both of these cases, the acceptor stem is the major identity determinant, and especially in the case of $tRNA^{Ala}$, it was shown that any wobble base pair introduced into the acceptor stem of the tRNA at the original wobble site conferred alanine specificity on the tRNA. Substantial alanine acceptor identity was retained when the original G-U wobble base pair was moved to another site in the acceptor helix (McClain et al., 1988).

It was also shown in one case that the accuracy of *in vivo* aminoacylation requires a proper balance of tRNA and aminoacyl-tRNA synthetase (Swanson et al., 1988). In another report, the modified base lysidine (see the section dealing with modified bases in the tRNA) was shown to be responsible for the proper aminoacylation of the $tRNA^{Ile}_{LAU}$ (Muramatsu et al., 1988).

Other functions of tRNA

In addition to its role in protein synthesis, tRNA is capable of participating in other diverse and fascinating reactions within the cell. Some of these special roles of tRNA include the following: several aminoacyl-tRNAs were shown to be involved in the regulation of specific amino acid biosyntheses (reviewed in Umbarger, 1980; Eisenberg et al., 1980). A $tRNA^{Leu}$ was reported to participate in branched-chain amino acid transport in both bacteria and mammalian cells (Quay and Oxender, 1980). Certain tRNA species ($tRNA^{Lys}$, $tRNA^{Pro}$, $tRNA^{Trp}$) were shown to act as the primers for reverse transcriptases (reviewed in Dahlberg, 1980). More recently, $tRNA^{Ser}_{7}$ of Drosophila was proposed for the same role in the replication of retrotransposons in Drosophila (Inouye et al., 1986; Cribbs et al., 1987a). In *E. coli*, a $tRNA^{Arg}$ species is required for DNA replication (Walker, 1987). Yet another new role for a barley chloroplast tRNA was proposed by Schon et al. (1986), who showed that a $tRNA^{Glu}$ is required for the conversion of glutamate to DALA (delta amino levulinic acid), a precursor of chlorophyll.

Suppression and the code

In addition to all these functions, tRNAs are capable of another more indirect role in translation. This involves their capability, in certain situations, of recognizing and suppressing missense, frameshift or nonsense mutations. Of the 64 possible codons in the genetic code, 61 correspond to one of the 20 amino acids and the other three (UGA, UAA, UAG) usually act as the codons specifying termination of protein synthesis (Garen, 1968). Rare exceptions to this rule occur in the genetic code of organelles and mycoplasmas, where, for example, UGA is read as tryptophan. Furthermore, in several systems, at low efficiency, the nonsense codons UGA and UAG can be misread by a normal charged tRNA ($tRNA^{Trp}$ and $tRNA^{Gln}$ respectively) by a translational readthrough mechanism (reviewed in Engelberg-Kulka and Schoulaker-Schwarz, 1988). Another recent example of this kind involves the modified amino acids selenocysteine and phosphoserine. Selenocysteine is incorporated into the mammalian enzyme glutathione peroxidase (Chambers et al., 1986) and the *E. coli* anaerobic enzyme formate dehydrogenase (Zinoni et al., 1986), in response to a UGA codon. It was found later that this is accomplished by a $tRNA^{Ser}_{UGA}$ in which the serine of the charged tRNA is converted to selenocysteine through exchange of the hydroxyl group of the serine with a selenol group (Leinfelder et al., 1988). The example of phosphoserine is discussed below (suppression in other eukaryotes).

Missense mutations are those where a point mutation changes the codon specifying one amino acid to that of another. If the presence of this different amino acid in the protein interferes with the activity of the protein, function can, sometimes, be restored by extragenic suppressor mutations that lead to the insertion of an acceptable amino acid into that position. The classic example of missense suppressors involves the *E. coli* tryptophan synthetase α protein system (reviewed in Hill, 1975; Murgola, 1985). In this system, missense suppressors are mutant $tRNA^{Gly}$ alleles which insert glycine in response to codons for other amino acids. The codon recognition properties of these missense

suppressors were changed either by nucleotide substitution in the anticodon ($tRNA^{Gly}$) or by nucleotide insertion in the anticodon loop, (outside of the anticodon) of the $tRNA^{Gly}$ (Prather et al., 1981; reviewed in Murgola, 1985). In some cases, the missense suppressor tRNAs were found to be misacylated. An example of this kind is $tRNA^{Lys}$, which is misacylated with glycine or alanine (Murgola and Pagel, 1983).

In the case of frameshift mutations, the correct amino acid reading frame is shifted due to the insertion or deletion of a base. Frameshift suppressors restore the reading frame by their ability to recognize codons of more or less than three bases (reviewed in Roth, 1974; Murgola, 1985). The classic examples of (+1) frameshift suppressors include sufA, sufB, sufC, sufD, sufE, and sufF in *Salmonella typhimurium*, which correct the insertion of a C or G residue in a stretch of C's or G's respectively. In the case of sufD, this was shown to be brought about by a $tRNA^{Gly}$ with 8 nucleotides (Riddle and Carbon, 1973) in the anticodon loop instead of the usual 7 nucleotides. It was suggested that the tetranucleotide anticodon CCCC might read a codon with four bases (GGGG) and thus restore the reading frame. A more recent work (O'Mahoney et al., 1989) reports the presence of a suppressor that leads to (-1) frameshift suppression in trpE91 mutation of *S.typhimurium*. This frameshift suppressor was identified to be $tRNA^{Gly}$ (sufS) in Salmonella, where frameshifting was shown to be achieved by doublet decoding. This suppressor causes a -1 shift at the zero-frame sequence CAG GGA GUG, resulting in insertion of the amino acids Gln Gly Ser, with the Ser being decoded from the sequence AGU. The base G immediately 5' of the GGA suppression site was shown to influence the level but was found to be not critical for suppression by sufS. From this result it was inferred that sufS causes frameshifting by doublet decoding and inserting glycine in response to the two base sequence GG.

Point mutations changing the codons specifying an amino acid to any one of the stop codons lead to the premature termination of translation. Nonsense suppressor tRNAs, recognize these stop codons and insert amino acids at those positions. The early

experimental evidence which identified the molecular basis for nonsense suppression is discussed by Garen (1968) and Korner et al., (1978). Suppression of nonsense mutations by altered tRNA molecules was reported to behave in a dominant manner, exhibiting allele specificity and gene nonspecificity (Garen,1968). Most of the classical nonsense suppressors in *E. coli* are tRNA mutants that arose by nucleotide substitution in the anticodon. An exception to this was reported by Hirsh (1971), where UGA suppression by a $tRNA^{Trp}$ in *E. coli* was found to involve an alteration in base 24 in the D stem rather than the anticodon. This base change conferred on this $tRNA^{Trp}$, the ability to recognize both UGA and UGG codons. Therefore, alteration in the anticodon is not the only way of obtaining nonsense suppressor tRNAs; albeit there is only this one exception to date.

Suppression of nonsense and frameshift mutations can also be achieved by altered components of the ribosomes (ribosomal or omnipotent suppression) in the absence of suppressor tRNAs. Ribosomes with altered proteins allow a certain level of codon misreading, leading to weak suppression. These altered proteins also modulate the efficiency of suppression by suppressor tRNAs. For example, *str A* and *ram* mutations in *E. coli* affect, respectively, the S12 and S4 proteins of the large ribosomal subunit. The *str A* mutations were shown to restrict the efficiency of suppression of nonsense and missense mutations (Gorini, 1970; Biswas and Gorini, 1972) while the *ram* mutations restored the efficiency of nonsense suppressors (Rosset and Gorini, 1969). Another example of this kind is found in *S. cerevisiae*. The omnipotent nonsense suppressors designated as sup1 and sup2 (or sup35 and sup45) (Hawthorne and Leupold, 1974) were found to be recessive suppressors of nonsense codons. The properties (for example, the spectrum of nonsense suppression and the interactions with dominant tRNA-mediated suppressors) of recessive suppressors suggested that these could also be altered components of the ribosome. However, the recent cloning (Himmelfarb et al., 1985; Breining et al., 1984) and sequencing (Breining and Piepersberg, 1986; Kushnirov et al., 1987) of the sup2 gene revealed that it is homologous to elongation factor genes (reviewed in Surguchov, 1988).

Based on the localization of the nucleotide binding domain(s) on the omnipotent suppressor gene products, it was suggested that the function of these omnipotent suppressors probably involves a proof reading process of codon-anticodon interactions (Surguchov, 1988). A similar function has been previously suggested for the bacterial elongation factor EF-Tu (Hopfield, 1974).

Suppression in bacteria was first documented in 1965 (Engelhart et al, 1965). A lot of our understanding of the phenomenon of suppression in prokaryotes comes from studies done in *E. coli*. Transfer RNA suppressors in *E. coli* have been obtained by single, double or triple base substitutions of the anticodon (reviewed in Eggertsson and Söll, 1988 and references therein), and all three kinds of nonsense suppressor tRNAs (amber, ochre and opal) have been identified. These are represented in Tables 1 and 2. The conventions used herein to represent the suppressor tRNAs are as described in Eggertsson and Söll (1988) for *E. coli;* Sherman (1982) for *S. cerevisiae*; Egel et al. (1980) for *S. pombe*; Hatfield (1985) for other eukaryotes; and Hodgkin et al. (1987) for *C. elegans*. For all the tRNAs, the wild type anticodon sequence is shown as the relevant subscript to 'tRNA'.

All of the opal suppressor tRNAs obtained in *E. coli* are also capable of reading the UGG (tryptophan) codon (reviewed in Eggertsson and Söll, 1988). The only known normal tRNA that acts as a suppressor tRNA and still possesses an unchanged anticodon, is the previously described $tRNA^{Trp}_{CCA}$ (Su-9) (the Hirsh suppressor). This result provided a striking example of the importance of tRNA conformation in codon recognition. All the ochre suppressor tRNAs in *E. coli* are capable of wobble reading the ochre as well as the amber nonsense codons. This is in striking contrast to the situation in yeast (described below) where strict codon specificity is exhibited by the ochre suppressors (Gesteland et al.,1976; Hottinger et al.,1984).

Most of the suppressor tRNAs in *E. coli* were obtained from tRNA genes present in more than one copy since, otherwise, the suppressor tRNAs would be expected to act as haplo-lethals. There are only four tRNA genes present in single copy from which

Table 1. Nonsense suppressors derived by single base substitutions in tRNA genes of *E. coli* (adapted and modified from Eggertsson and Soll, 1988).

tRNA	Gene	Suppressor symbol	Suppressor type	Obtained in vivo/in vitro	References
$tRNA_{CUG}^{Gln}$	gln V	sup E	Amber	*In vivo*	Inokuchi et al., 1979
$tRNA_{CAA}^{Leu}$	leu X	sup P	Amber	*In vivo*	Thorbjarnardottir et al., 1985; Yoshimura et al., 1984
$tRNA_{CGA}^{Ser}$	ser U	sup D	Amber	*In vivo*	Steege, 1983; Steege and Horabin, 1983; Thorbjarnardottir et al., 1985
$tRNA_{CCA}^{Trp}$	trp T	sup U	Amber	*In vivo*	Raftery et al., 1984 Söll, 1974; Yaniv et al., 1974
$tRNA_{GUA1}^{Tyr}$	tyr T	sup F	Amber	*In vivo*	Goodman et al., 1968
$tRNA_{GUA2}^{Tyr}$	tyr U	sup Z	Amber	*In vivo*	Petursdottir et al., (unpubl.)

Table 1. Continued. Nonsense suppressors derived by single base substitutions in tRNA genes of E. coli (adapted and modified from Eggertsson and Soll, 1988) .

tRNA	Gene	Suppressor symbol	Suppressor type	Obtained In vivo/In vitro	Referencces
$tRNA_{UUG}Gln$	gln U	sup B	Ochre	*In vivo*	Ozeki et al., 1980
$tRNA_{UUC}Glu$	glt T	glT (SuUAA/G)	Ochre	*In vitro*	Raftery & Yarus, 1985
$tRNA_{UUU}Lys$	lys T	sup L	Ochre	*In vivo* (spont:/DES)	Eggertsson & Adelberg 1965; Prather et al., 1983; Yushimura et al., 1984a
$tRNA_{UUU}Lys$	lys V	sup N	Ochre	*In vivo*	Uemura et al., 1985
$tRNA_{GUA1}Tyr$	tyr T	sup C	Ochre	from sup F by HA	Altman et al., 1971
$tRNA_{GUA2}Tyr$	tyr U	sup M	Ochre	*In vivo* (DES)	Eggertsson&Adelberg 1965; Orias et al.,1972 Eisenberg&Yarus,1980

Table 1. Continued. Nonsense suppressors derived by single base substitutions in tRNA genes of E. coli (adapted and modified from Eggertsson and Soll, 1988) .

tRNA	Gene	Suppressor symbol	Suppressor type	Obtained In vivo/In vitro	Referencces
$tRNA_{UCC}^{Gly}$	gly T	glyT (SuUGA)	Opal	*In vivo*	Prather et al., 1981
$tRNA_{CCA}^{Trp}$	trp T	trp T (SuUGA)	Opal	*In vivo*(EMS)	Söll, 1974; Raftery et al., 1984
$tRNA^{CCATrp}$	trp T	Su-9	Opal	*In vivo*	Hirsh, 1971

Table 2. Nonsense suppressors derived by double or triple base substitutions in tRNA genes of *E. coli* (adapted and modified from Eggertsson and Soll, 1988)

tRNA	Genes	Suppressor symbol	Suppressor type	Obtained *in vivo/in vitro*	References
$tRNA_{UCC}^{Gly}$	gly T	glyT(SuUAG-8)	Amber	*In vivo*/EMS	Murgola et al. 1983
$tRNA_{CCC}^{Gly}$	gly U	glyU(SuUAG)	Amber	*In vivo*	Murgola, 1985
$tRNA_{VGA}^{Ser}$	ser T	serT(SuUAG)	Amber	*In vitro*	Rogers & Soll. (unpubl.)
$tRNA_{CUG}^{Gln}$	gln V	glnV(SuUAA/G)	Ochre	*In vivo* (from supE)	Inokuchi et al. 1979 Ohlsson et al. 1968
$tRNA_{UCC}^{Gly}$	gly T	glyT(SuUAA/G)	Ochre	*In vivo* (from glyT amber	Murgola et al. 1984
$tRNA_{CCC}^{Gly}$	gly U	glyU(SuUAA/G)	Ochre	*In vivo*	Murgola, 1985
$tRNA_{GCC}^{Gly}$	gly V	glyV(SuUAA/G)	Ochre	In vivo/NG	Murgola & Jones, 1978
$tRNA_{CAA}^{Leu}$	leu X	leuX(SuUAA/G)	Ochre	*In vitro*(from supP)	Murgola et al. 1978 Rogers & Soll, (unpubl.)

Table 2. Continued. Nonsense suppressors derived by double or triple base substitutions in tRNA genes of *E. coli* (adapted and modified from Eggertsson and Soll, 1988) .

tRNA	Genes	Suppressor symbol	Suppressor type	Obtained *in vivo*/*in vitro*	References
$tRNA_{CGA}^{Ser}$	ser U	serU(SuUAA/G)	Ochre	*In vivo*&*In vitro* (from supD)	Person & Osborn, 1968; Murgola & Jones, 1978
$tRNAc_{CA}^{Trp}$	trp T	supV	Ochre	*In vivo* (EMS)	Söll & Berg, 1969 Söll, 1974; Raftery et al 1984
$tRNA_{CCC}^{Gly}$	gly U	glyU(SuUGA)	Opal	*In vivo*	Murgola, 1985
$tRNA_{GCC}^{Gly}$	gly V	glyV(SuUGA)	Opal	*In vivo*/Spont.	Murgola, 1981 Murgola, 1985

suppressor derivatives were obtained. These are $tRNA^{Ser}_{CGA}$(supD), $tRNA^{Leu}_{CAA}$(supP), $tRNA^{Gly}_{UCC}$ [glyT(SuUGA)] and $tRNA^{Trp}_{CCA}$[supU and trpT(SuUGA)]. In the case of $tRNA^{Ser}_{CGA}$ the lethality problem was obviated by the presence of $tRNA^{Ser}_{VGA}$ an isoacceptor capable of wobble reading other serine codons (Grosjean et al, 1985). Even though $tRNA^{Leu}_{CAA}$ also has an isoacceptor ($tRNA^{Leu}_{UAA}$) the latter cannot fully compensate for the former's ability to read the UUG codon. This leads to slow growth rate in the $tRNA^{Leu}_{CAA}$ amber suppressor carrying strains (Thorbjarnardottir et al., 1985a; Yamiazumi et al., 1980; reviewed in Eggertsson and Söll, 1988). Suppressor $tRNA^{Gly}_{UCC}$ retains some of its ability to read the GGA and GGG codons *in vivo* (Murgola and Pagel, 1980), which is sufficient for survival. The suppressor allele of $tRNA^{Trp}_{CCA}$ was originally obtained in a partial diploid (Söll and Berg, 1969). The suppressor mutation in the $tRNA^{Trp}_{CCA}$ amber suppressor (supU), changes its charging specificity so it inserts glutamine as well as tryptophan in a 9:1 ratio (Knowlton, Söll and Yarus, 1980; Knowlton and Yarus, 1980; Celis et al., 1976; Söll and Berg, 1969).

Recently, studies of suppression in *E. coli* have focussed on the construction of a complete set of amber suppressor tRNAs, one for each of the 20 naturally occurring amino acids (reviewed in Eggertsson and Söll, 1988). Reports indicate construction of 12 such amber suppressor tRNAs (Normanly et al., 1986a and b).

Transfer RNA suppressors in yeast, as in *E. coli*, are derived from multicopy genes (see Sherman, 1982 for a review). The only recessive lethal suppressor reported so far is SUP-RL1, which inserts serine in response to UAG codons (Brandriss et al., 1976). The lethality was shown to be due to the loss of a $tRNA^{Ser}$ which has a unique decoding function (Etcheverry et al., 1982). The yeast suppressor tRNAs that have been most extensively studied are UAA and UAG suppressors. In *S. cerevisiae* (Sherman et al. 1979), the iso-1-cytochrome c system was used to establish the amino acids inserted by each of these suppressors. Most of such yeast suppressors were found to insert either tyrosine (SUP2-8, SUP 11), serine (SUP 16-19 and SUP 22, SUP-RL1) or leucine (SUP

26-29, SUP 32,33, SUP52-56). In total, 8 tyrosine-inserting suppressors (corresponding to the eight tyrosine tRNA genes), 5 serine-inserting suppressors (corresponding to 5 serine tRNA genes), and 11 leucine-inserting suppressors were found. In the case of the last group, 7 of them were shown to map to known $tRNA^{Leu}$ gene loci. Information about the remaining four is still lacking. For all of the tyrosine-inserting suppressors, both UAA and UAG allelic forms (allelic suppressors) have been found. For SUP16, SUP17, SUP19 and SUP22 (serine inserting suppressors) only ochre alleles are available. The only serine-inserting amber suppressor is SUP-RL1, which is a haplo-lethal. Ochre suppressing alleles are available for SUP26 to SUP33, while only amber suppressing alleles are available for SUP52 to SUP56 (both leucine-inserting suppressor groups). The efficiency of suppression was found to be high for those inserting tyrosine, low to moderate for serine-inserting suppressors and low to very low for leucine-inserting suppressors. The suitability of the amino acid at a particular position specific for each nonsense allele and/or the codon context effect may be responsible for these differences in efficiency of suppression. Some of the yeast suppressor tRNAs have been shown to exhibit strict codon specificity of suppression of Qβ RNA and globin mRNA nonsense codons in *in vitro* protein synthesizing systems. These include: the UAA suppressors SUP 4-o (Gesteland et al., 1976), SUP 16-o, SUP 17-o and SUP 19-o (Ono et al., 1981; Broach et al., 1981; and C. Waldon et al., cited in Sherman, 1982); and the UAG suppressors SUP 6-a, SUP-RL1-a (Gesteland et al., 1976), SUP7-a, and SUP 16-a (Capecchi et al., 1975). For most of these, it has also been proven by nucleotide sequencing that they do have tRNAs that are altered at the anticodon.

Suppression of the ade 5,7-143 allele was used as the basis to identify the first UGA suppressors in *S. cerevisiae* (Hawthorne, 1976). The UGA suppressors isolated by suppression of this allele include SUP71-80, and SUP 85-90. Although the tRNAs associated with these suppressors were not established, close linkages of these with leucine or serine inserting UAA suppressors were indicated (Sherman, 1982). The only known

examples of *S. cerevisiae* suppressor tRNAs that insert amino acids other than tyrosine, leucine or serine include the ochre and amber tRNA suppressor genes derived from $tRNA^{Phe}_{GAA}$ by gene synthesis (Masson et al., 1987) and opal and amber alleles of tryptophan tRNA synthesized *in vitro* by site-directed mutagenesis of the anticodon of the gene (A. Atkin et al., personal communication; Kim and Johnson, 1988).

In the fission yeast, *S. pombe*, all three (opal, ochre and amber) suppressor tRNAs have also been identified (Janner et al., 1979; Kohli et al., 1980; Krupp et al., 1985; reviewed in Egel et al., 1980). Both opal and ochre alleles of sup 3, sup 9, sup 12, sup 8, and sup 10 have been described, in which the former three (sup 3, 9 and 12) are all serine-inserting tRNAs (Rafalski et al., 1979; Mao et al., 1980; Hottinger et al., 1982; Willis et al., 1984), while sup 8 and 10 insert leucine (Wetzel et al., 1979; Kohli et al., 1980; Sumner-Smith et al., 1984). The first amber suppressor tRNA (sup SI) was described by Krupp et al. (1985) which is a $tRNA^{Ser}_{AGA->CUA}$ mutant. In the same work, sup 13 and sup 14 were also identified as amber suppressor tRNAs.

The efficiency of suppression, as measured by read through of nonsense codons, is dependent on the mRNA reading context. Studies done in T4 rIIB, T4 lysozyme, *E. coli* β-galactosidase and two *E. coli trp* genes (Salser, 1969; Salser et al., 1969; Yahata et al., 1970; Comer et al., 1974; Comer et al., 1975; Colby et al., 1976; Akaboshi et al., 1976; Fluck et al., 1977; Feinstein and Altman, 1977; Feinstein and Altman, 1978) using homotopic (UAA and UAG mutations of the same codon) nonsense sites revealed that the UAA/UAG suppression ratios vary over a ten fold range. This finding confirms that mRNA reading context must play a role in nonsense codon recognition. For most of the cases, amber suppression was more efficient, while at others UAA suppression was favored. Of course, if we measure efficiency by restoration of function, the suitability of a particular amino acid in restoring protein function is also a factor.

More recently, the context effect has been quantitated in studies of suppression of different *E. coli* lacI amber and opal mutations using a fusion of lacI and lacZ genes (Bossi,

1983; Miller and Albertini, 1983). In that study, the efficiency of amber suppression was measured at 42 sites (Miller and Albertini, 1983), and considerable variation was found among the studied amber suppressors (sup D, E, F, and P) in their ability to produce suppression at these sites. The range of variation was highest (35 fold) for the weakest suppressor supE. For others, only a 7 to 10 fold range of variation was found.

Many attempts have been made to rationalize observed differences in the efficiency of suppression with features of mRNA or tRNA structure. There is no clear cut evidence favoring either explanation, and suppression may finally depend on individual situations. A comparison of sequences around the anticodon revealed that efficiency may depend on the sequences 3' to the codon in the message. It was found that suppression was more efficient if the base in the mRNA 3' to the codon was a purine (Bossi,1983). However, exceptions to this rule are also reported, where efficient suppression was found if the codon was followed by CUC or CUG in the message (Miller and Albertini, 1983). It has been suggested that the sequence of the anticodon loop and stem must 'match' the 3' end nucleotide of the anticodon for efficient suppression (Yarus, 1982). The disharmony sometimes created between this 'cardinal nucleotide' of the anticodon (by mutational change to a suppressor) and the anticodon loop and stem might lead to weak or no suppression. Recently, this effect was demonstrated in the case of an ochre suppressor $tRNA^{Glu}$ in *E. coli*. The ochre suppressor $tRNA^{Glu}$ is a very inefficient suppressor, created by a base change of C to A at the 3' nucleotide of the anticodon. It was shown that as the similarity of the anticodon stem and loop was increased (by mutational alteration) towards that of a tRNA that recognizes UXX codons, so did the efficiency of suppression (Raftery and Yarus, 1987).

There are also examples in the literature where mutations outside of the anticodon stem/loop structure of the tRNA gene affect the efficiency of suppression. The reduced efficiency is found to be a direct result of the effect of the mutations on the processing and/or maturation of tRNA. In the case of sup3 $tRNA^{Ser}$ of *S. pombe*, fifteen types of

point mutations within the gene locus were found which reduced or abolished the suppression of nonsense codons by this tRNA (Pearson et al., 1985). The suppressor activity and 5' end processing of an *S. pombe* opal suppressor (sup9-e) were shown to be affected by an intron mutation, which destabilized the secondary structure (Willis et al., 1986). Intron mutations have also been shown to affect the splicing of the SUP53 precursor tRNA of *S. cerevisiae* (Strobel and Abelson, 1986). The *in vivo* suppression by tRNAs carrying these intron mutations was shown to reflect their splicing phenotype, and thus decrease or even abolish suppression, depending on the type of mutation. Another example of this kind was demonstrated in the case of the opal suppressor $tRNA^{Trp}$ of *S. cerevisiae* in our laboratory, where an anticodon change alone (without a corresponding intron change) was shown to prevent efficient processing and suppressor activity of the suppressor tRNA (Atkin et al., personal communication).

Nucleotide modifications in and around the anticodon may also affect the efficiency of suppression (Bjork, 1984). In *E. coli*, the *mia* A mutation functions as an antisuppressor mutation. It was shown to prevent formation of the i^6A residue 3' to the anticodon, and lead to reduced amber suppression (Gefter and Russel, 1969; Petrullo et al., 1983). In *S. pombe*, also, an antisuppressor mutation was shown to affect the post-transcriptional modification of the wobble base in the anticodon (Heyer et al., 1984). In this case, the *sin-3* mutation was found to result in loss of the nucleoside 5-(methoxycarbonylmethyl)-2-thiouridine from the wobble position of the tRNAs, which was correlated with a reduced efficiency of suppression by the UGA and UAA suppressor $tRNA^{Ser}$ and suppressor $tRNA^{Leu}$. Another example of this kind was provided by Johnson and Abelson (1983), for the yeast ochre suppressor $tRNA^{Tyr}$ gene where the intron was shown to be essential for correct modification of the anticodon sequence, which in turn is required for efficient suppression by its gene product.

Some suppressor tRNAs have deleterious effects on their hosts. One of these effects is on the growth rate. Ochre suppressors in *E. coli* often affect growth rates and the

growth inhibition is most pronounced for the strong ochre suppressors (eg. supC, supM). This inhibition probably reflects the frequent usage of UAA as a termination codon but there is no direct evidence for read through of natural terminators in ochre suppressor carrying strains. Unlike the ochre suppressors in *E. coli*, amber suppressors do not usually affect growth rate in supplemented media (Gorini, 1970). There is one example in *E. coli* of an amber suppressor causing read through of an identified protein resulting in a significant effect on cell physiology (Gallant, 1979). This protein is the product of the rel A gene (stringent factor) that controls the stringent response. Strains carrying strong amber suppressors produce an extended, apparently inactive stringent factor, which results in a relaxed stringent response. In *S. cerevisiae*, strong suppressors are always associated with slow or impaired growth (reviewed in Sherman, 1982) and, contrary to the case in *E. coli*. it is the amber suppressors which frequently have a more deleterious effect (Liebman and Sherman, 1976).

Suppression in other eukaryotes

Though most of our understanding of eukaryotic suppression has its basis on studies done in yeast, there are several reports of suppression in other eukaryotes (Hatfield, 1985 and other references therein). These include suppression in nematodes (Bolten et al., 1984; Waterston and Brenner, 1978; Fire, 1986; reviewed in Hodgkin et al., 1987), Neurospora (Seale et al., 1977), mouse cell lines (Kuchino et al., 1987), and bovone cell lines (Diamond et al., 1981; Hatfield et al., 1982). Suppression in the higher eukaryotes was often demonstrated either by introduction of suppressor tRNAs (Cremer et al., 1979; Capecchi et al., 1977; Bienz et al., 1980; Bienz et al., 1981), or the genes encoding suppressor tRNAs (Laski et al., 1982; 1984; Temple et al., 1982; Hudziak et al., 1982; Young et al., 1983; Summers et al., 1983) from other organisms into eukaryotic cells or, cell lines.

Among eukaryotes other than yeast, the most information about nonsense suppression is known for the nematode *C. elegans*. The initial suppressors discovered were sup-5 and sup-7 (Waterston and Brenner, 1978; Waterston, 1981). These were shown to suppress an amber mutation of the myosin gene. This was demonstrated *in vitro* using the suppressor tRNA in a heterologous translation system (Wills et al., 1983) and *in vivo* by microinjection of suppressor tRNA into amber mutant animals (Kimble et al., 1982). The sup-7 gene was sequenced (Bolten et al., 1984) and shown to encode a $tRNA^{Trp}$ with an anticodon of CUA. Since then, other amber suppressors (sup24, sup28, sup29) have been described (reviewed in Hodgkin et al., 1987) and all of them including sup5 were found to be altered forms of $tRNA^{Trp}$ genes. Differences in efficiencies were found among these amber suppressor tRNAs: sup-7 was found to be the most efficient and sup-29 was the least efficient. A recent study revealed that the suppressor genes were differentially regulated in a tissue and developmental stage-specific manner (Kondo et al., 1988).

Suppressors of chain termination mutations have been described in another eukaryote *Neurospora crassa* (Seale et al., 1977). The mutation *am(17)* in the structural gene for NADP-specific glutamate dehydrogenase was suppressed by insertion of either tyrosine or leucine at residue 313, a position occupied in the wild type gene by glutamic acid. Both types of insertion were shown to restore the activity of the enzyme, but no definite association with a tRNA molecule was made in that study.

The above studies led to the identification of several opal, ochre and amber nonsense mutations in these eukaryotes (Cremer et al., 1979; Capecchi et al., 1977), and also demonstrated that suppression is dependent on the context effect (Bienz et al., 1981) and that suppression of termination codons is not necessarily detrimental to eukaryotic cells in culture (Laski et al., 1984; Hudziak et al., 1982). In the nematode, however, the animal was barely able to tolerate two copies of the strongest sup-7 suppressor, and it was suggested that a suppressor gene expressed at more than twice the level of sup-7 would be

lethal (Waterston, 1981), presumably as a result of readthrough of natural terminators. There have also been reports of natural suppression by misreading of termination codons by normal cytoplasmic aminoacyl tRNAs (see Hatfield, 1985 for a review). These include: $tRNA^{Trp}$ from rabbit reticulocytes (Geller and Rich, 1980); $tRNA^{Tyr}$ from Drosophila (Bienz and Kubli, 1981); $tRNA^{Tyr}$ from tobacco (Beier et al., 1984); and $tRNA^{Ser}$ from mammalian, avian and Xenopus tissues (Diamond et al., 1981; Hatfield et al., 1982). The basis for suppression in these instances is either due to absence ($tRNA^{Tyr}$) or presence ($tRNA^{Ser}$) of a specific modified base in the wobble position of the anticodon. There are also reports of the presence of a naturally occurring opal suppressor $tRNA^{Ser}$ with an anticodon of TCA in the chicken (Hatfield et al., 1983), and human (O'Neill et al., 1985) genomes. The conservation of homology in this tRNA species between the chicken and human genomes led to speculations about these naturally occurring suppressor tRNAs having specialized cellular functions. One suggestion was that these suppressor tRNAs respond to UGA codons in a specific codon context and insert phosphoserine into the protein (reviewed in Hatfield, 1985).

Suppressors in heterologous systems

As mentioned before, the promoter sequences of tRNA genes in eukaryotes are found within the coding sequences for the mature tRNA, and are also relatively highly conserved (Sprinzl et al., 1989; reviewed in Sharp et al., 1985). In addition, there are several instances where 5' and/or 3' flanking sequences provide necessary or important information for modulating tRNA gene transcription rates (reviewed in Sharp et al., 1985). The internal control sequences are sufficiently conserved to allow deduction of a consensus promoter sequence (Galli et al.,1981) while the regulatory information in the flanks tends to be species specific (Sprague et al., 1980; Sharp et al., 1985). The transcriptional and processing machinery for tRNA genes is similar enough among eukaryotes, that *in vitro* and *in vivo* expression of heterologous tRNA genes is often

possible (Nishikura et al.,1981; Hottinger et al., 1982; Laski et al., 1984; Capone et al., 1985). However, the placement of a tRNA gene from one species into the genome of another via transformation techniques does not always result in functional expression of these heterologous tRNA genes. For example, cloned suppressor tRNA genes from *S. pombe* function as codon-specific nonsense suppressors when introduced into *S. cerevisiae* (Hottinger et al., 1982; 1984), but *S. cerevisiae* suppressor tRNA genes did not function in *S. pombe* when the reciprocal experiment was done (Krupp et al., 1985). Other *S. pombe* suppressors, however, do not function properly in *S. cerevisiae* (Gamulin et al., 1983; Sumner-Smith et al., 1984). Furthermore, there is evidence that the same *S. pombe* $tRNA^{Ser}_{UGA}$ gene that functions in *S. cerevisiae* does not function when introduced into *D. melanogaster* (Molnar et al., 1988).

Although not enough examples have been characterized to give an unequivocal explanation as to why some tRNA genes function in a heterologous milieu while others do not, failure to be transcribed efficiently or spliced has sometimes been invoked (Sumner-Smith et al., 1984; Krupp et al., 1985; Dingermann et al.,1988). Informational suppression in *S. cerevisiae* and *S. pombe* is codon-specific (Gesteland et al., 1976; Sherman., 1982; Hottinger et al., 1984), although in some genetic backgrounds, this can be circumvented (Gelugne and Bell, 1988). Also, when yeast tRNA suppressors are introduced into *E. coli*, this codon specificity is lost (Rossi et al., 1982). Normally, however, when heterologous suppressor tRNA genes are introduced into *S. cerevisiae*, the codon specificity is retained (Hottinger et al., 1982; 1984).

Suppression in Drosophila

Although there have been examples of suppression in Drosophila, there have not been many examples of tRNA-mediated nonsense suppression in this organism. Historically, suppressor of sable [*su(s)*] was considered as a prime candidate for a nonsense suppressor. Suppressor of sable, in homozygotes, was involved in the

suppression of some alleles of pigment mutants, like sable, speck, purple and vermilion; thus conforming to the properties of informational suppressors which are expected to be allele specific and gene nonspecific (Garen,1968). Most of our understanding of the *su(s)* locus comes from work done on vermilion mutants (Twardzik et al., 1971; Rizki and Rizki, 1963). It was known that in vermilion mutants, the enzyme tryptophan pyrrolase was defective (Rizki and Rizki, 1963) leading to the accumulation of tryptophan (Green, 1949). Twardzik et al. (1971) found that the levels of tryptophan were reduced in *v/v; su(s)/su(s)* files. They also found differences in the chromatographic profiles of tyrosyl tRNAs between the wild type and *su(s)* homozygotes. Of the two separable $tRNA^{Tyr}$ species in the wild type, one was found to inhibit the enzyme tryptophan pyrrolase (Jacobson, 1971), suggesting that the $tRNA^{Tyr}$ was, in some way, involved in the suppression of the vermilion mutation. This led to the idea that this $tRNA^{Tyr}$ could be acting as a suppressor tRNA. However, this was later disproved by the findings of White et al. (1973), who demonstrated that the differences in the chromatographic profiles of tyrosyl tRNA are only due to the levels of the modified nucleoside Q. The absence or reduced levels of this modified base in the $tRNA^{Tyr}$ of *v/v; su(s)/su(s)* homozygotes led to the loss of differences in the chromatographic profiles of this species of tRNA in the *su(s)/su(s)* flies. This undermodification was suggested to relieve simultaneously the allosteric inhibition conferred by this species of tRNA on the tryptophan pyrrolase, thus restoring wild type eye color in the *v/v; su(s)/su(s)* flies. It is still not completely clear as to how the suppression was effected in this case.

The Present Investigation

The search for true nonsense suppressors in Drosophila continues. In our laboratory, using previously produced putative nonsense mutants (Girton et al., 1979) of the structural gene for xanthine dehydrogenase (Xdh), a screen was done to detect second site revertants that restore XDH activity. Though this screen produced revertants, no

candidates for suppressor tRNAs were identified. In another laboratory, a similar search was conducted unsuccessfully among 21 genetically characterized suppressor stocks (Bienz and Kubli, 1981). Later, a wild type $tRNA^{Tyr}$ with the anticodon GψA was identified as a natural suppressor of the leaky UAG codon of the tobacco mosaic virus RNA (Bienz and Kubli, 1981), but no tRNA fraction isolated from the suppressor stocks was shown to contain any suppressor activity.

About the same time, nonsense mutations were identified in Drosophila in the structural genes for alcohol dehydrogenase (Adh) (Kubli et al., 1982), and the flight muscle actin gene, Act88F (Karlik et al., 1984) (both trp to UGA mutations). Furthermore, the technique of germline transformation of Drosophila using P-element vectors became available (Rubin and Spradling,1982; Spradling and Rubin,1982). These discoveries shifted the emphasis towards introducing extant heterologous suppressor tRNA genes into the genome of Drosophila by germline transformation. In our laboratory the sup3e $tRNA^{Ser}_{UGA}$ gene (opal suppressor) from *S.pombe* was introduced in this manner into the Drosophila genome (Molnar et al.,1988). Suppressor activity was not detected. The advent of the technique of the oligonucleotide directed, *in vitro* mutagenesis (Zoller and Smith.,1982), switched the emphasis of the search to *in vitro* synthesis of Drosophila suppressor tRNA genes, by mutagenesis of the anticodon. These suppressor tRNA genes were introduced into appropriate tester strains containing nonsense alleles of *Adh*. Three tRNA species were chosen for these studies ($tRNA^{Tyr}$, $tRNA^{Leu}$, and $tRNA^{Ser}$) based on the natural suppressor tRNAs found in yeast, the most extensively studied eukaryote with respect to informational suppression. Studies with $tRNA^{Tyr}$ and $tRNA^{Leu}$ were continued by Dr. Kubli's group in Switzerland and Dr. Hartl's group in St. Louis respectively while our emphasis has been on Drosophila serine tRNA.

As mentioned above, initial work done in our laboratory involved the sup-3e $tRNA^{Ser}$ gene from *S. pombe* and introducing it into the Drosophila genome (Molnar et al., 1988) by P-element mediated germline transformation (Rubin and Spradling, 1982;

Spradling and Rubin, 1982). The transformed lines were crossed with the CyO^{nB} (*nB*=opal allele of Adh) strain and assayed for ADH acitivity, but no ADH activity was found. This failure could be due to any one of several reasons. The simplest one being that the gene, from a heterologous system, was not being transcribed and processed well.

The present study was undertaken with the objective of finding out if that was, indeed, the case. A $tRNA^{Ser}$ gene from *D. melanogaster* was chosen for this study. Serine tRNAs recognize six codons of two classes: UCN and AGPy, and at least three different serine tRNAs (with anticodons IGA, CGA and GCU) are required for this (Cribbs et al., 1987a). The serine tRNA gene family of *D. melanogaster* is a complex group, consisting at least seven members. Some of these were fractionated using benzoylated-DEAE cellulose columns, and shown to decode AGC, AGU ($tRNA_2^{Ser}$), UCG ($tRNA_4^{Ser}$); AGU, AGC ($tRNA_5^{Ser}$); UCG ($tRNA_7^{Ser}$) in ribosome binding assays (White et al., 1975). In some instances, these results were confirmed by nucleotide sequencing of the tRNAs (for $tRNA_{2b}^{Ser}$, $tRNA_4^{Ser}$, $tRNA_7^{Ser}$) (Cribbs et al., 1987a). The $tRNA_{UCN}^{Ser}$ family ($tRNA_4^{Ser}$ and $tRNA_7^{Ser}$) contains about 11 or 12 genes, of which eight are located on the X chromosome (band 12DE) and another one is located at 23E on the left arm of the second chromosome ($tRNA_7^{Ser}$, the subject of this thesis). Among the eight genes found on the X-chromosome, extensive microheterogeneity was reported by Cribbs et al. (1987b). Among these, different classes of sequences were found. Four of the eight genes correspond to $tRNA_4^{Ser}$ or $tRNA_7^{Ser}$ (which are 96% homologous); two were reported to be the result of single cross over between $tRNA_4^{Ser}$ and $tRNA_7^{Ser}$, one a double cross over product, and the last one was reportedly different from $tRNA_4^{Ser}$ by a single C to T transition at position 50 (Cribbs et al., 1987b). The $tRNA_7$ gene chosen for this study encodes a major serine tRNA species (White et al., 1975) and is known to be transcribed well *in vitro* (Sajjadi and Spiegelman.,1989). This gene was previously cloned into the *Hin*dIII site of the pBR322 vector (Dunn et al., 1979). A recombinant DNA clone of this $tRNA_7^{Ser}$ gene was obtained from Dr. Tener (University of British Columbia) and its anticodon region was altered by

oligonucleotide-directed site-specific mutagenesis (Kunkel, 1985; Zoller and Smith, 1982). In this manner, a repertoire of suppressor alleles that recognize the opal, ochre and amber nonsense codons was constructed. These were later tested for activity in a heterologous system (*S. cerevisiae*) as well as in a homologous system (Drosophila).

This thesis consists of: the construction of the above suppressor tRNA genes by site-specific mutagenesis, transformation of *S. cerevisiae* with these constructs, a demonstration of the suppression of various nonsense mutant alleles of yeast by these constructs in a codon-specific manner, a demonstration of active transcription of these suppressor tRNA genes in the heterologous yeast system, successful transformation of *D. melanogaster* with these tRNA constructs, characterization of the transformed lines, and preliminary studies done to understand the basis for the lack of suppression of the nonsense alleles of Drosophila genes by these suppressor tRNA gene constructs.

Chapter II

MATERIALS AND METHODS

1. Strains Used:

Three strains of the bacterium *Escherichia coli* were used during the course of this research.

a). HB101 (Boyer et al., 1969; Bolivar and Backman, 1979): F⁻ *leu*B6 *pro*A2 *rec*A13 *thi*1 *ara*14 *lac*Y1 *gal*K2 *xyl*5 *mtl*1 *rps*L20 λ⁻*sup*E44 *hsd*S20 rB⁻ mB⁻. This is a laboratory strain maintained as a glycerol stock at -20° C. The original source for this strain is unknown. It was used as a host for plasmids conferring ampicillin resistance on the host.

b). JM105 (Yanisch-Perron et al., 1985): *thi rps*L *end*A *sbc*B15 *hsp*R4 Δ(*lac-pro*AB) (F' *tra* D36 *pro*AB *lac*Iᑫ ZΔM15). This strain was kindly provided by Dr. K.L.Roy. It was used as a host in transformation experiments involving M13 phage vectors or their derivatives.

c). BW313 (Kunkel, 1985): Hfr KL16 *Pol*45 (*Lys*A(61-62)) *thi*1 *rel*A1 *spo*T1 *dut*1 *ung*1. This strain was obtained on a plate from Dr. Linda Reha-Krantz. It was used as a host in preparing deoxyuridine-containing single stranded DNA templates for site-directed mutagenesis of the $tRNA_7^{Ser}$ gene of *Drosophila melanogaster*.

A single strain of *Saccharomyces cerevisiae* was used as a recipient in the transformation experiments described herein.

d). JG 369-3B (α): *ade*2-1, *lys*2-1, *can*1-100 (UAA alleles), *met* 8-1, *trp*1-1 (UAG alleles), *leu*2-2, *his*4-260 (UGA alleles), *ura*3-52(non-suppressible, selectable marker). This strain was a gift from Dr. Jean Paul Gelugne and was used for functional tests of the *in vitro* constructed nonsense-suppressor $tRNA^{Ser}$ genes of Drosophila in a heterologous system.

2. Plasmids/phage vectors:

(i) pDt5: a derivative of pBR322 containing a 4.4 kb *Hin*dIII Drosophila DNA fragment, having the coding sequence for the $tRNA^{Ser}_7$ gene of *D. melanogaster*.

Source: Dr. G.M. Tener.

(ii) M-PH1: This is a derivative of M13mp18 phage vector containing a 2.4 kb *Pst*I-*Hin*dIII Drosophila DNA fragment. This fragment contains the wild type $tRNA^{Ser}_7$ gene.

Source: This work

Use: This was used as a source of the wild type $tRNA^{Ser}_7$ gene for the site-directed mutagenesis experiments.

(iii) M-OL4, M-OC1 and M-AM1: These are derivatives of M-PH1 in which the anticodon of the $tRNA^{Ser}$ gene was changed from AGA to TCA, TTA or CTA such that the mature product of these genes would recognize the opal, ochre or amber nonsense codons, respectively.

Source: This work

Use: These were used as the source of the mutant constructs for cloning into the yeast and Drosophila transformation vectors.

(iv) YRp7 (Tschumper and Carbon, 1980): An *E. coli*-yeast shuttle vector with ampicillin resistance as the selectable marker in *E. coli* and tryptophan prototrophy as the selectable marker in yeast.

Source: Dr. J.B. Bell

Use: This was used as a vehicle for introducing the *in vitro* constructed nonsense-suppressor $tRNA^{Ser}$ genes into *Saccharomyces cerevisiae*.

(v) pSS166, pSS152, pSS179 and pSS182: These are derivatives of YRp7 that contain the wild type $tRNA^{Ser}_7$ gene from *D. melanogaster* or its opal, ochre and amber derivatives respectively, (cloned into the *Sal*I site of YRp7).

Source: This work

Use: These are used to test for the biological activity of suppression, by the *in vitro* constructed Drosophila nonsense suppressor tRNA genes, in *S. cerevisiae*.

(vi) Carnegie20 (Rubin and Spradling, 1983): An *E. coli*-Drosophila shuttle vector with ampicillin resistance as the selectable marker in *E. coli*. It also contains a crippled P-element with intact ends which facilitates the introduction of foreign and/or *in vitro* manipulated DNA sequences into the genome of *Drosophila melanogaster*.

Source: Dr. J.B. Bell

Use: This was used as a vehicle to introduce the *in vitro* constructed $tRNA^{Ser}$ genes into *D. melanogaster*.

(vii) pSS193, pSS06, pSS11f and pSS35: These are derivatives of Carnegie20 containing the wild type $tRNA^{Ser}$ gene or its opal, ochre and amber derivatives respectively.

Source: This work

Use: These are used to test for the biological activity of suppression of the *in vitro* constructed $tRNA^{Ser}$ genes in the mileau of the entire Drosophila genome.

(viii) pπ25.7 wc (Rubin and Spradling, 1983; Karess and Rubin, 1984): This is a derivative of pπ25.7, containing a P-element with intact coding sequences but with one crippled inverted repeat.

Source: Dr. R.B. Hodgetts

Use: This was used as a helper P-element to provide transposase in the Drosophila transformation experiments. This cannot integrate by itself due to a crippled end repeat required for integration.

3. *Drosophila melanogaster* stock list:

(i) *b cn; ry^8*:

Description: black (body color mutation), cinnabar, rosy 8 (both eye color mutations)

Source: Dr. J.B. Bell

Use: This was used as a recipient in the Drosophila transformation experiments.

(ii) *CyO^{nB} pr cn/Adh fn^7 pr cn; ry^{a9}/ry^{a9}:*

Description: Curly-Oster, null B allele of Adh, purple, cinnabar/fn^7 allele of Adh, purple, cinnabar with a9 null allele of rosy.

Source: Dr. J.B. Bell

Use: The null B allele of Adh is an opal mutant and so was used to test for the ability of the *in vitro* constructed opal suppressor $tRNA^{Ser}$ gene to suppress.

(iii) *Adh^{n4}; ry:*

Description: black, n4 null allele of Adh, rosy

Source: Dr. M. Ashburner

Use: The n4 allele of Adh is an amber mutant and so was used to test the activity of the *in vitro* constructed amber suppressor $tRNA^{Ser}$ genes.

(iv) (a) wt4, (b) op1, (c) oc3, (d) am5, am8, am13:

All the above fly stocks were derived from *b cn; ry^8* and contain respectively an extra wild type $tRNA^{Ser}$ gene (a),or its opal (b), ochre (c), and amber (d) suppressor derivatives, integrated into the genome of Drosophila through P-element mediated, germline transformation. The stock 'wt4' is derived from line 'W24' in Table 6; 'op1' is derived from line 'S36' in Table 7; 'oc3' is derived from line 'C28' in Table 8; and 'am5', 'am8', and 'am13' are derived from lines 'M16', 'M26' and 'M39' in Table 9 respectively.

Source: This work

Use: (a), (b), and (d) were used as donors in crosses made to transfer the suppressor tRNA genes from an Adh^+ background to an Adh^- (*nB or n4*) background. All of them were also used as a source of DNA for genomic Southern hybridizations.

(v) 'op1 Cy WT' and 'am13 Cy WT':

Complete genotype: $\frac{CyO^{nB}\ pr\ cn}{Adhn4}\ ;\ \frac{ry}{ry}\ (ry+)$

Source: this work

Use: These strains were constructed to bring the Adh nonsense allele and the suppressor tRNA genes (opal/amber respectively) into the same genetic background. The (ry^+) designation indicates that the fly contains the introduced transposon with the ry^+ selectable marker, but does not specify anything about the possible genomic location of the transposon.

(vi) 'op1 Cy ry' and 'am13 Cy ry': The genotype of these strains is the same as above, with the exception that the transposon carrying the suppressor tRNA gene is absent.

Source: This work

Use: These were used as negative controls to the stocks in (v) in suppression assays.

4. Growth media

All strains were grown at 37° C (for *E. coli*) or 30° C (for yeast) on the various media as specified below.

(i) Bacteria: *E. coli* strains HB101 and BW313 were grown on standard LB media containing 10 g Tryptone, 10 g sodium chloride, 5 g yeast extract and 12 g Bacto-agar per liter of distilled water (Maniatis et al.,1982). When necessary, ampicillin was added to the media at a concentration of 50-100 µg/ml.

E. coli strain JM105 were grown on M9+thiamine medium (Maniatis et al.,1982). containing 6.0 g Na_2HPO_4, 3.0 g KH_2PO_4, 0.5 g NaCl, 1.0 g NH_4Cl, 12.0 g bacto-agar. 2.0 ml of 1M $MgSO_4$, 10.0 ml of 20% glucose, 0.1 ml of 1M $CaCl_2$ per liter of distilled

water. Filter-sterilized thiamine-HCl was added at the rate of 10 ml of 10 mg/ml stock solution per liter.

For M13 transformations, a soft agar overlay was used. The soft agar consisted of Bactotryptone (10 g/l), yeast extract (5.0 g/l) NaCl (5.0 g/l), glucose (1.0 g/l) and Bacto-agar (7.0 g/l).

(ii) Yeast: Yeast strains were grown at 30° C on either YEPD or the appropriate omission media. YEPD is a complete medium consisting of 20 g glucose, 10 g yeast extract, 20 g Bactopeptone per liter. Bacto-agar (10 g/liter) was added to make solid media. The omission media consisted of the YM (yeast minimal) medium (7 g of yeast nitrogen base without amino acids, and 20 g of dextrose per liter), with the appropriate amino acid supplements using the recipe of Sherman et al. (1986).

(iii) All the Drosophila stocks were grown on standard fly food prepared as in Nash and Bell (1968).

5. Maintenance and storage of stocks:

Strains of *E. coli* and yeast were maintained at 4° C and sub-cultured every 2-3 weeks onto fresh media. For long term storage, glycerol stocks were prepared as in Maniatis et al. (1982) and stored at -20° C in tightly capped and sealed vials.

All the fly stocks were maintained either in standard-sized vials or bottles at room temperature (22° C). The stocks were transferred to fresh media once every 2 weeks.

6. 'Minipreparations' of plasmid DNA:

Plasmid DNAs were prepared on a small scale for quick analysis as described in Birnboim and Doly (1979), with some modifications. Ten ml cultures were grown to saturation overnight, in the appropriate media at 37° C with vigorous shaking. Bacterial cells were harvested by centrifugation at 7000 rpm for 10 min. The cell pellet was resuspended in 200 µl of buffer (50 mM glucose, 25 mM Tris-HCl, pH 8.0 and 10 mM

EDTA) and transferred to an Eppendorf microcentrifuge tube. Four hundred microliters of freshly prepared alkaline lysis solution (0.2 M NaOH and 1% SDS) were added to the tube, and gently mixed by inversion. The tube was kept on ice for 5 min, and 300 µl of cold 3 M sodium acetate (pH 4.8) solution were then added and mixed well. The tube was left for 10 min. on ice and then centrifuged for 10 min. in a microcentrifuge at full speed (14,000 rpm). The supernatant was transferred to a fresh tube, 0.6 volumes of 2-propanol added, kept at -20° C for five minutes and centrifuged for ten minutes. The supernatant was decanted and the last traces of liquid carefully removed. The pellet was dissolved in 200 µl of a 50 mM Tris-HCl (pH 8.0), 0.15 M NaCl solution followed by two phenol extractions, one phenol-chloroform isoamyl alcohol (50:48:2) extraction and one chloroform isoamyl alcohol extraction. The DNA was finally precipitated by addition of two volumes of 95% ethanol followed by centrifugation and the pellet was washed twice with 70% ethanol. Each time, after adding 70% ethanol, the tube was kept on ice for 30 min. followed by a five minute centrifugation. The pellet was dried under vacuum and finally dissolved in 50 µl of TE (pH 8.0). The DNA was stored at -20° C. The small scale DNA preparations were treated with pancreatic RNase (10 mg/ml) during restriction endonuclease digestions, to hydrolyze any RNA in the preparations.

7. Small scale preparations of RF DNA from M13 derived clones:

A 10 ml culture of LB was inoculated with a fresh overnight culture of JM105, and infected with phage from a single plaque. The flask was incubated for 7 hrs at 37° C with vigorous shaking. The cells were pelleted by centrifuging for 5 min. at 5,000 rpm., resuspended in one ml of Tris-Sucrose buffer (25% sucrose, 50 mM Tris-HCl, pH 8.0 at 4° C), and 250 µl of lysozyme solution (10 mg/ml in 250 mM Tris-HCl, pH 8.0) were added. The contents of the tube were gently mixed and kept on ice for 15 minutes. Then 0.5 ml of 0.25 M EDTA was added, mixed, incubation on ice continued for 15 more min., followed by the addition of 2 ml of lysis buffer (2% SDS, 50 mM Tris-HCl, pH 8.0, and

62.5 mM EDTA), a further 15-60 min. on ice, the addition of one ml of 8 M potassium acetate, and a further 30 min. on ice. The debris was pelleted by centrifuging the tube for 10 min. at 12,000 rpm, the supernatant transferred to a fresh Eppendorf tube, 0.3 volumes of 8 M ammonium acetate and two volumes of 2-propanol added, mixed, kept at room temperature for ten min., and centrifuged for ten min. at 10,000 rpm. The supernatant was carefully discarded, and the pellet was resuspended in 300 µl of H-NET buffer (100 mM Tris-HCl, pH 7.8 at 4° C, 150 mM NaCl, and 1 mM EDTA). To this, 10 µl of DNase-free RNase (10 mg/ml) were added, incubated at 37° C for 30 min., followed by addition of 10µl of proteinase K (3 mg/ml), and a further incubation of 30 min. at 37° C. The contents of the tube were extracted once with buffer-saturated phenol, followed by one extraction with phenol:chloroform (50:48:2) and two extractions with chloroform:isoamyl alcohol (24:1). The aqueous phase was collected and precipitated with 0.5 vol. of 8 M ammonium acetate and two volumes of 95% ethanol. The DNA was dissolved in 200 µl of TE and precipitated once more using 95% ethanol. The pellet was finally dried under vacuum and dissolved in 50 µl of TE. The DNA thus prepared was stored at -20° C.

8. Large scale isolation of plasmid/RF DNA

Plasmid and M13 RF DNAs were isolated from 500 ml cultures of the appropriate medium (LB with the antibiotic ampicillin for plasmids, and M9+thi medium for M13 derived RFs) using the alkaline lysis procedure of Maniatis et al. (1982). All the P-element and M13 derivatives were grown to saturation without chloramphenicol and others were all amplified using chloramphenicol as described by Maniatis et al. (1982).

(i) Plasmid DNAs

Overnight cultures were grown to saturation in an appropriate medium at 37° C with vigorous shaking, in a volume of 5 ml. Two hundred µl of the overnight culture were added to 5 ml of fresh medium and incubation continued at 37° C for nine more hours. After 9 hrs., the 5 ml were added to 500 ml of fresh medium and incubated for 12-16 hrs at

37° C. If amplification was desired, 2.5 ml of a solution of chloramphenicol (34 mg/ml in ethanol) were added to a 500 ml culture at an A_{600} of 0.4-0.6, and incubation continued at 37° C, with vigorous shaking, for a further 12-16 hrs.

(ii) M13 derived DNAs

A 10 ml culture of JM105 was grown to saturation in M9+thi, at 37° C. This overnight culture was used to prepare competent cells of JM105, as described in section 12 (ii). The competent cells (200 µl) were transformed with either 1.0 µl (0.1-0.5 µg) of RF DNA or with 1.0 µg of single-stranded DNA. After transformation, 0.8 ml of fresh LB medium was added and the tube incubated at 37° C. for 30 min., without shaking. The entire contents of the tube were then added to 500 ml of M9+thiamine medium, that was pre-incubated at 37° C for approximately one hour in the presence of about one ml of fresh overnight culture of JM105. The incubation at 37° C was continued for a further 12-16 hrs., with vigorous shaking.

After the 12-16 hr. incubation, harvesting, and the isolation of plasmid/RF DNA from all of the above was done by alkaline lysis as described in Maniatis et al. (1982). All the DNA preparations were further purified by cesium chloride-ethidium bromide isopycnic gradient centrifugation as per Maniatis et al. (1982). After ultracentrifugation, the ethidium bromide was removed by several extractions with 1-butanol, saturated with TE (pH 8.0) buffer. The DNA was then precipitated overnight in four volumes of 70% ethanol, followed by a reprecipitation in 2 volumes of 95% ethanol. The DNA thus prepared was quantitated as in Maniatis et al. (1982) and stored frozen at -20° C.

9. Restriction endonuclease digestions of DNA

Restriction digests were performed essentially as described in Maniatis et al. (1982). A typical restriction endonuclease digestion reaction consisted of the following: x µl of DNA (plasmid/RF/genomic), 5 µl of 10x buffer, 2 µl of 0.1 M spermidine

(Bouche,1981), y µl of restriction endonuclease, z µl of sterile double-distilled water in 50 µl of total reaction volume.

All the components except the restriction endonuclease were added to a 1.5 ml Eppendorf tube, and the tube was kept on ice for a few min. before the addition of enzyme (Bouche, 1981). After incubation at 37° C (or the appropriate temperature for an enzyme), for approximately two hours, a second aliquot of restriction endonuclease was added and incubation continued for a further 2 hrs (for plasmid or RF DNA) or overnight (for genomic DNA). After digestion, the enzyme was inactivated by incubating at 68° C for ten minutes. The digested DNA was then analyzed by electrophoresis on agarose gels.

10. Agarose gel eleotrophoresis and elution of DNA fragments

Routinely, 0.7-1.0% agarose gels were used for both analytical and preparative purposes. The gels were cast and used as in Maniatis et al. (1982). After electrophoresis, the gels were stained in 100 ml of double distilled water containing 0.5 µg of EtBr/ml for about ten minutes, and photographed.

Two methods were used to isolate desired DNA restriction fragments from agarose gels. Initially, LMP agarose gels were used for this purpose, and later DNA was electroeluted onto a Whatman 3MM paper backed by a dialysis membrane. The procedures used were exactly as described in Maniatis et al. (1982).

11. Ligations

Before ligations, the purified restriction fragment and the linearized vector were spin-dialysed together (Maniatis et al.,1982), using 500 ml of TE (pH 8.0) buffer, for 30 min. at room temperature. The spin dialysis membrane (0.02 µm pore size, Millipore) was floated on the surface of the TE buffer. The vector and the insert were mixed together in a ratio of 1:5 and the drop was gently placed on the surface of the membrane. Spin dialysis was continued for 30 min., while slowly stirring the buffer. After the spin dialysis was

completed, ligations were done by mixing x µl of vector-insert mixture, 1.0 µl of 10x ligase buffer (Maniatis et al. 1982), 1.0 µl of 0.1 M DTT, 0.3 units ligase (BRL), y µl distilled water in 10 µl of total reaction volume. All the ligation reactions were carried out overnight at 14^0 C, at which time they were used to transform competent host cells.

12. Transformation of *E. coli*

Different methods were used to prepare competent cells of the two *E. coli* host strains used. Fresh competent cells were prepared for each transformation.

(i) Competent cells of HB101

An overnight culture of HB101 was grown to saturation at 37° C in LB broth, with vigorous shaking. A 200 µl aliquot of the fresh overnight culture was used to inoculate 10 ml of fresh LB medium and grown at 37° C until an A_{600} value of 0.4-0.6 was reached. The flask was placed on ice for 30 min., cells centrifuged for 10 min. at 5000 rpm, the pellet resuspended in a half volume of 50 mM chilled calcium chloride, left on ice for a further 30-60 min, and the cells pelleted by centrifugation as above. The supernatant was discarded, the cell pellet was resuspended in one tenth volume of cold 50 mM calcium chloride, and kept on ice until further use.

(ii) Competent cells of JM105

A fresh overnight culture of JM105 was grown in 5 ml of M9+thiamine medium by vigorous shaking at 37° C. A 0.1 ml aliquot of the overnight culture was inoculated into 10 ml of YT broth and incubated at 37° C until an A_{600} value of 0.4-0.6 was reached. The culture was cooled on ice for five to ten minutes, cells collected by centrifuging at 5000 rpm for 10 min., and the pellet gently resuspended in 5 ml of transformation mix #1 (100 mM NaCl, 5 mM $MgCl_2$ and 5 mM Tris-HCl, pH 7.6). The cells were again pelleted and gently resuspended in 5 ml of transformation mix #2 (100 mM $CaCl_2$, 250 mM KCl, 5 mM $MgCl_2$ and 5 mM Tris-HCl, pH 7.6). The cells were kept on ice for 30 min. to 24 hours,

pelleted as above and resuspended in 1 ml of transformation mix #2. The cells were kept at 4° C or on ice and used up to 24 hours later.

(iii) Transformations and plating

Competent cells prepared as above were used as transformation hosts. Typically, 200 µl of competent cells were placed in a 1.5 ml Eppendorf centrifuge tube, 1.0 to 5.0 µl DNA added, kept on ice for 30 min. and then heat-shocked at 42° C. for 2 min. For ampicillin resistant plasmid DNA, 0.8 ml of LB was added to the tube after heat shock, kept at 37° C for about 45 min. without shaking, followed by centrifugation for 20 sec. at 14,000 rpm., resuspension in 50-100 µl of LB, spreading onto an LB+amp plate and incubation at 37° C overnight. For transformations with M13 DNA, the following were added to the cells in a 5 ml glass tube after the heat shock: 10 µl of IPTG (25 mg/ml in distilled water), 50 µl of Bluo-gal (25 mg/ml in N,N-dimethylformamide), 200 µl of log phase JM105 cells, and 3.0 ml of soft agar. The contents were mixed by quickly vortexing and poured onto a YT plate. When the soft agar hardened, the plates were incubated overnight at 37° C.

13. Single-stranded DNA isolation from clones of M13 origin

A 5 ml culture of JM105 was grown to saturation in M9+thiamine medium at 37° C. A 100 µl aliquot of this overnight culture was used to inoculate 25 ml of YT medium, along with a 'colorless' plaque, and grown at 37° C for 7 hours. The cells were pelleted by centrifuging at 18,000 rpm for 30 min., and the supernatant was immediately decanted into a clear 50 ml polypropylene centrifuge tube. One ml of supernatant was kept frozen at -20° C as a source of phage stock. To the remaining supernatant, 6.5 ml of (10% PEG8000, and 2.5 M NaCl) solution was added, mixed and kept at 4° C overnight. The phage were then pelleted by centrifuging at 10,000 rpm for 10 min., resuspended in 650 µl of phenol extraction buffer (0.3 M NaCl, 0.1 M Tris-Cl, pH 7.8 at 4° C, 1 mM EDTA), and transferred to a fresh Eppendorf tube. Ten µl of 10% SDS and 10 µl of proteinase K

(3 mg/ml) solutions were added to the tube, mixed, incubated for 30 min. at 37° C, followed by two extractions with buffer-saturated phenol, one extraction with equal volumes of phenol-chloroform-IAA (50:48:2) and three extractions with chloroform:IAA (24:1). The DNA in the final aqueous phase was precipitated with 95% ethanol, followed by two washes with 70% ethanol. The pellet was finally dried, dissolved in 25 µl of distilled water, and stored frozen at -20° C.

14. Dideoxy sequencing of M13 clones

The M13 clones were sequenced using the dideoxy chain termination method of Sanger et al. (1977).

(i) Primer-template annealing reaction

About 0.5-1.0 µg of DNA was placed in an Eppendorf tube with 5.0 ng of the 'a14' primer (described in Fig.6 of Results) and 1.3 µl of 10x Polymerase buffer (70 mM Tris-Cl, pH 7.5, 70 mM $MgCl_2$ and 50 mM NaCl). The volume was made up to 13 µl with distilled water and mixed. The tube was kept at boiling temperature for three min. and slowly cooled to room temperature for about one hour.

(ii) Sequencing reaction

To the tube containing the annealed primer and template, 1.0 µl of 0.25 M DTT, 1.0 µl of [α–^{32}P]dATP or [α–^{35}S]dATP and 1.0 unit of Klenow (large) fragment of DNA polymerase I were added and mixed. From this tube, 3.0 µl was added to each of the four tubes labeled A,C,G and T. To each tube, 3.0 µl of appropriate reaction mixture (described below) were added. The contents were mixed, incubated for 15 min. at 37° C., 1.0 µl of chase-Klenow (1 mM each of dNTP with 1.0 unit of Klenow fragment) added to each tube, incubated at 37° C for a further 15 min., and 14 µl of formamide dye (made by adding 10 mg Bromophenol Blue, 10 mg of xylene cyanol, 200 µl of 0.5 M EDTA and 9.7 ml of deionized formamide) were added to each tube to terminate the reaction. After boiling for three minutes, two microliters of each reaction were loaded onto a 6% sequencing gel .

(iii) Reaction mixes

(a) for [α–^{32}P]dATP

The 'A' reaction mix contained 42 µM each of dCTP, dGTP and dTTP, 8.5 µM of ddATP and 1.4x polymerase buffer. The 'C' reaction mix contained 42 µM each of dGTP, and dTTP, 6.75µM dCTP, 67.5 µM of ddCTP and 1.4x polymerase buffer. The 'G' mix contained 42 µM each of dCTP and dTTP, 6.75 µM dGTP, 135 µM ddGTP and 1.4x polymerase buffer. The'T' mix contained 42 µM each of dCTP and dGTP, 6.75 µM dTTP, 265 µM dTTP and 1.4x polymerase buffer.

(b) for [α–^{35}S]dATP

The 'A' reaction mix contained 38 µM each of dCTP, dGTP and dTTP, 20 µM ddATP and 1x polymerase buffer. The 'C' mix contained 6 µM dCTP, 54 µM each dGTP and dTTP, 300 µM ddCTP and 1x polymerase buffer. The 'G' mix contained 6 µM dGTP, 54 µM each of dCTP and dTTP, 300 µM ddGTP and 1x polymerase buffer. The 'T' mix contained 6 µM dTTP, 54 µM each of dCTP and dGTP, 600 µM ddTTP and 1x polymerase buffer. The mixes and stock solutions were stored at -70° C. The recipes for all the above mixes were obtained from Dr. F.E. Nargang.

(iv)Polyacrylamide gel electrophoresis of the sequencing reactions

A 6.0% polyacrylamide (38:2 acrylamide to bis-acrylamide) gel, containing 8.3 M urea and 100 mM TBE (pH 8.3), was used for analyzing the sequencing reactions. A 40 ml aliquot of gel solution was mixed with 250 µl of 10% ammonium persulfate and 10 µl of TEMED and quickly poured between glass plates separated by 0.3 mm thick spacers. The slot former was placed between the plates, and the gel allowed to polymerize for a period of two hours to overnight at room temperature. After polymerization, the slot former and the bottom spacer were removed, and the gel was fitted onto a vertical gel apparatus (Tyler Research Corporation). The gel was pre-run at 35 watts of constant power for 30 min., then the reactions were loaded and electrophoresed for the required

length of time at a constant power of 35 Watts. Whenever [α–^{32}P]dATP was used, one of the glass plates was removed after the electrophoresis and the gel wrapped in Saran wrap and exposed to Kodak X-Ray film overnight at -45° C. However, with [α–^{35}S]dATP, the gel was lifted off onto Whatman 3MM paper, covered with Saran wrap, and dried for 3 hours under vacuum at 80° C on a gel drier (Bio-Rad). The dried gel was then exposed to Kodak X-Ray film overnight at -45° C.

15. Double stranded template sequencing

Plasmid derivatives of YRp7 and Carnegie20 were sequenced using double stranded plasmid DNA as the template using the method of Chen and Seeburg (1985).

(i). 'Minipreparations' of plasmid DNA for double stranded template sequencing

The procedure used was the modified alkaline lysis method of Maniatis et al. (1982). A 5 ml culture of the appropriate strain was grown at 37° C, with antibiotic. The cells were pelleted by centrifuging for five minutes, resuspended in 1.5 ml STE (100 mM NaCl, 10 mM Tris-Cl, pH 7.5 and 1 mM EDTA), transferred to an Eppendorf tube, and the cells were pelleted again. The pellet was resuspended in 200 µl of cold TGE (50 mM glucose, 25 mM Tris-Cl, pH 8.0 and 10 mM EDTA) containing 5 mg/ml lysozyme and the cells kept on ice for 5 min. To the tube, 400 µl of freshly prepared alkaline lysis solution (0.2 N NaOH and 1% SDS) were added, gently mixed and left on ice for five min. An aliquot of 300 µl of cold 3 M potassium acetate, pH 5.8 (made by adding 60 ml of 5 M potassium acetate, 11.5 ml glacial acetic acid and 28.5 ml of distilled water) was then added to the tube, mixed sharply, and kept on ice for a further 10 min. The tube was centrifuged for five min. at 4° C., supernatant transferred to a clean tube, boiled for one min., and placed in an ice water bath. After one minute, the tube was centrifuged for 5 min. at room temperature, the supernatant transferred to a clean Eppendorf tube with 750 µl of isopropanol, mixed well and centrifuged for 10 min. The pellet was dissolved in 200 µl of distilled water and 200 µl of 5 M lithium chloride, 50 mM MOPS, pH 8.0 solution were

added. The tube was placed on ice for 15 min. and centrifuged for 5 min. To the supernatant, 900 µl of 95% ethanol were added and the DNA pelleted by centrifuging for 10 min. The pellet was dried and resuspended in 100 µl of TE, 1 µl of DNase-free RNase A (10 mg/ml) was added, and the tube was incubated at 37° C for 15 min. Ten µl of 3M sodium acetate were added and extracted once with 100 µl of phenol-chloroform:IAA (50:48:2). The final aqueous fraction was ethanol precipitated, washed twice with 70% ethanol, and the pellet dissolved in 50 µl of distilled water after drying.

(ii) Sequencing

(a) Denaturation of template and annealing to the primer:

Plasmid DNA (8-16 µg) was placed in an Eppendorf tube and 1/10 vol. of 10x denaturant (2M NaOH and 2 mM EDTA) was added, incubated at room temperature for five min. Then, 1/10 vol. of 2M ammonium acetate and 2.5 vol of 95% ethanol were added to the tube and it was kept for 5 min. at -20° C. The DNA was pelleted by centrifuging for 10 min. and the pellet dried and dissolved in 8 µl of annealing mix (25 mM Tris-Cl, pH 8.5 and 15 mM $MgCl_2$). A 5-20 ng quantity of primer was added to the tube and incubated at 37° C for 15 min.

(b) Sequencing reaction:

A 7.5 µl aliquot of sequencing mix (13 mM DTT, 13 mM Tris-Cl, pH 8.5), 1.0 µl of (2-4 units) of Klenow fragment, and 1.0 µl (10 µCi) of [α–^{35}S]dATP were mixed together. Two µl of this mixture were added to each of the 1.5 ml Eppendorf tubes labeled A, C, G and T. Two µl of the primer-template mix and 2 µl of appropriate deoxy/dideoxy mixtures were also added to the tubes, and incubated for 20 min. at 42° C. Two µl of dNTP chase mix (described under section 14.ii), were added to each tube and incubated for an additional 5 min., followed by the addition of 5 µl of formamide dye mix (described under section 14.ii.). The sequencing reactions were electrophoresed on a 6% acrylamide, 8.3 M urea, 100 mM TBE gel as described in section 14. iv.

(iii) Reaction mixes for double stranded sequencing

All the mixes were made in 10 mM Tris-Cl, pH 7.5. The 'A' mix contained 150 μM each of dCTP, dGTP and dTTP, as well as 16.25 μM of ddATP. The 'C' mix contained 10.5 μM dCTP, and 214 μM each of dGTP and dTTP as well as 45 μM ddCTP. The 'G' mix contained 214 μM each of dCTP and dTTP and 10.5 μM dGTP as well as 100 μM ddGTP. The 'T' mix contained 214 μM each of dCTP, dGTP, and 10.5 μM dTTP as well as 264 μM ddTTP.

16. Oligonucleotide-directed *in vitro* mutagenesis

The procedure used was a combination of the methods of Kunkel (1985) and Stewart et al. (1985) with some modifications.

(i) Preparation of single-stranded DNA template containing uracil (Kunkel, 1985).

An overnight culture of BW313 (*dut ung*) was grown to saturation at 37° C, with vigorous shaking. A 200 μl aliquot of the fresh overnight culture was inoculated into 10 ml of YT medium and was grown at 37° C until the culture reached late log phase. Different dilutions (10^{-4} to 10^{-12}) of the frozen phage stock of the appropriate M13 derivative were plated onto YT medium along with BW313 log phase cells. The plates were incubated at 37° C overnight. The next day, single plaques were inoculated into 25 ml YT medium containing 0.5 μg/ml uridine along with 200 μl of fresh BW313. The culture was grown at 37° C for 7 hrs with shaking. The cells were pelleted and single-stranded DNA templates prepared as described in section 13.

(ii) Mutagenesis

The double priming procedure of Stewart et al. (1985) was used for the *in vitro* mutagenesis. The primer kinasing reaction contained 2 picomoles of 'a14' oligonucleotide upstream primer (described in Fig. 6) and 4 picomoles of the mutant oligonucleotide primer, 0.8 μl of 10x kinase buffer (100 mM $MgCl_2$, 100mM Tris-Cl, pH 7.5 and 1 mg/ml

gelatin), 0.8 µl of 0.06 M β-mercaptoethanol, 1.0 µl of 10 mM ATP, and 1.0 unit of T4 polynucleotide kinase in a total volume of 8 µl. The tube was incubated at 37° C for 45 minutes and then kept at 65° C for 10 minutes. For the primer-template annealing reaction, 1.0 µl of 0.5 M NaCl and 1.0 µl (0.05 pmoles) of M13 single-stranded DNA were added to the tube containing the kinased primers. The tube was heated at 85° C for 2 min. and cooled to room temperature slowly for about one hour. For the primer extension reaction, 10.0 µl of solution B (20 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM each of dNTPs, and 1 mM ATP), 0.2 units of ligase and 1.0 unit of Klenow were added. The tube was then incubated at 37° C for 30 sec. and at 14° C overnight. The next day, 5.0 µl of the primer extension reaction were used to transform competent JM105 cells, as described in section 12(iii). The plaques obtained were analysed by plaque hybridizations to the mutant primer.

17. Plaque hybridizations to nitrocellulose filters

The prpcedure of Benton and Davis (1977) was used for plaque hybridizations. The plates containing plaques were cooled for at least one hour at 4° C. Nitrocellulose filters (Millipore, HATF) were gently laid on the agar surface for one minute, lifted off, laid plaque side up on a Whatman 3MM paper soaked in a denaturing solution (0.15 M NaOH, 0.5 M NaCl), and left for about 60 sec. The filter was then treated twice with neutralizing solution (0.5 M Tris-HCl, pH 7.5 and 0.5 M NaCl), for 60 sec. each time, washed for 10 min. in 2x SSC (1x SSC: 0.15 M sodium chloride, and 0.015 M sodium citrate), air-dried for 30 min., and baked for 2 hours at 80° C under vacuum. The filters were then hybridized to the appropriate end-labeled oligonucleotides.

The filters were pre-hybridized for 2 hrs. at 42° C in 6x SSC, 5x Denhardt's reagent and 0.5% SDS in the presence of 150 µg of *Azotobacter* DNA per ml. End-labeled oligonucleotide probe was then added to the bag and hybridized overnight at 42° C, with

gentle shaking. After 12-16 hrs. of hybridization, the filters were washed once in 4x SSC, 0.1% SDS at 42° C for 10 min. and exposed to a Kodak X-Ray film at -45° C.

18. Colony hybridizations to nitrocellulose filters

The procedure used was essentially the same as described in section 17, except that the colonies on the filters were denatured for 5 min. in 0.5 N NaOH, 0.5 M Tris-Cl, pH 8.0 and neutralized in 0.5 M Tris-Cl, pH 8.0, and 1.5 M NaCl.

19. Radiolabeling of DNA

Oligonucleotides that were 5' end-labeled with [γ-^{32}P]ATP (4500 Ci/mmol) or DNA fragments primed with random hexanucleotides and labeled with [α-^{32}P]dCTP were used for all the hybridizations during the course of this research.

(i). 5' End-labeling of oligonucleotides:

The labeling or kinasing reaction contained 10 picomoles of oligonucleotide, 2-3 µl of (20-30 µCi) of [γ-^{32}P]ATP, 2 µl of 10x kinase buffer (described in section 16. ii.), 2 µl of 60 mM β-mercaptoethanol and 5 units of T4 polynucleotide kinase in a total reaction volume of 20 µl. The tube was incubated at 37° C for 45 min. and the reaction was stopped by the addition of 2 µl of 0.5 M EDTA pH 8.0. Whenever end-labeled oligonucleotides were used as probes in Northern and Southern hybridizations, the unincorporated nucleotides were removed from the probes by passing them through a Sephadex G-25 column, as described in Maniatis et al. (1982).

(ii) Labeling DNA using random-hexanucleotides and [α-^{32}P]dCTP.

This method of labeling was used to make probes for Southern (Southern, 1975) hybridizations to genomic DNA. The procedure used was as described by Feinberg and Vogelstein (1983 and 1984). Plasmid DNA was digested with the appropriate restriction endonuclease(s) and electrophoresed on a 1% LMP agarose gel. When sufficient resolution was achieved, the desired restriction fragment was cut out of the gel and kept in

an Eppendorf tube. Sterile distilled water was added to the gel piece (1.5 ml/gm gel). The tube was kept at boiling temperature for 7 minutes, and then stored at -20° C. Just before the labeling reaction, the tube was re-boiled for 3 min. and kept at 37° C until required. The labeling reaction contained 10 µl of OLB mix, 2 µl BSA (10 mg/ml) up to 32.5 µl of DNA in agarose (20-100 ng), 5 µl of [α-^{32}P]dCTP (50 µCi), and 2 units of Klenow fragment in a total reaction volume of 50 µl. The tube was incubated at 37°C from 3 hours to overnight, depending upon the amount of DNA used in the reaction. After the labeling reaction, 50 µl of distilled water were added to the tube and the unincorporated nucleotides removed by passing through Sephadex G-50 column, as described in Maniatis et al. (1982). The OLB mix was made as described in Feinberg and Vogelstein (1984).

(iii)Nick Translation:

Nick translated probes were used for *in situ* hybridizations. The reaction was set up (Maniatis et al. 1982) by mixing x ul DNA (1-2 µg), 5 µl 10x nick translation buffer, 2 µl of dNTP mix (0.033 mM each dNTP, except the labeled dNTP), 0.5 µl DNase I (10 ng/µl), 0.5 µl *E. Coli* DNA Polymerase I (2.5 units), y µl distilled water, and 10 µl ^{3}H-dTTP (vacuum dried to zero volume) in 50.0 µl of final volume. The reaction was incubated at 15° C for 2-3 hours and stopped by addition of 60 µl of nick translation stop buffer (1% SDS and 50 mM EDTA). The probe was then purified over a Sephadex G-50 column, as described in Maniatis et al. (1982).

20. Transformation of yeast:

Yeast transformations were done using the method of Klebe et al. (1983). An overnight culture of the appropriate yeast strain was grown at 30° C in YEPD medium, and was inoculated into 10 ml of fresh YEPD to an A_{600} nm of 0.15/ml. The culture was incubated at 30° C, with shaking, until an A_{600} of 0.6 was obtained. The cells were pelleted by centrifuging for 3 min. at 3000 rpm and resuspended in 5 ml of SBEG (1 M sorbitol, 10 mM bicine, pH 8.3 and 3% ethylene glycol). The cells were again pelleted as

before and resuspended in 0.2 ml SBEG. Finally, the cells were incubated at 30° C for 5 min., with gentle shaking, and were ready to use in transformations. Fifty µl of cells were mixed with 0.5-1.0 µg of DNA and incubated at 30° C for 10 min. The cells were then frozen at -70° C for 20 min. and quickly thawed in a 37° C waterbath, with shaking. A 375 µl aliquot of PB (10% PEG 1000, and 0.2 M bicine, pH 8.3) was added and briefly homogenized. The tube was then incubated at 30° C for 1 hr without shaking, followed by addition of 500 µl of NB solution (150 mM NaCl, and 10 mM bicine, pH 8.3). The NB solution was added dropwise and gently mixed. The cells were pelleted in a microfuge for 20 sec. at 5000 rpm. The supernatant was removed, cells resuspended in 200 µl of fresh NB solution, plated onto selective medium, and incubated at 30° C.

21. Germline transformation of Drosophila

The germline transformations of Drosophila were done as described by Karess (1985) and Spencer (1987).

(i) Preparation of flies

Six bottles of the appropriate genotype were started every six to seven days. After the flies had laid eggs for about 5-6 days, all the parent flies were discarded, and the emerging progeny flies were saved. At about two to three days of age, all the flies were transferred to a small population cage, and kept in the dark. The flies were fed with regular fly food (Nash and Bell., 1968), in petri dishes (60x20 mm), with fresh yeast paste added. The flies were kept in the cage for two days before collecting embryos. Flies of 4-8 days of age were used to produee the embryos, and the embryos were gathered after one hr. of egg deposition onto grape agar plates.

(ii)Preparation of DNA solution

All materials (Eppendorf tubes, pipette tips etc.) that come in contact with DNA and injection buffer were cleaned with distilled water, and 95% ethanol, air-dried and autoclaved. The vector DNA and helper DNA (px 25.7 wc) were mixed in an Eppendorf

tube in the ratio of 1:1.2 molecules, and ethanol precipitated. A typical precipitation reaction consisted of approximately 11.2 µg of construct DNA, 5.0 µg of helper DNA, 1/10 vol. of 3M sodium acetate, and 2.5 vol of 95% ethanol. The tube was kept at -70° C for 1 hr, centrifuged for 15 min. at room temperature, washed once with 70% ethanol in 0.2 M NaCl, and once with 70% ethanol. The DNA was finally redissolved in 50 µl of injection buffer (5 mM KCl, 0.1 mM sodium phosphate, pH 6.8). All the solutions were pre-spun immediately before use. The DNA in the injection buffer was stored at -20° C.

(iii) Preparation of slides

Regular cytology slides and coverslips were prepared for injections. A strip of double sided sticky tape was placed in the centre of a slide, and a coverslip on top of it. Two thin strips of double-sided sticky tape were placed on the coverslip at either end, for lining up the embryos. The slides were stored in a dust-free environment.

(iv) Preparation and loading of needles

Drummond Microcaps (25µl) were used to prepare needles using an electric needle puller. The settings of the needle puller were empirically determined. The needles were stored by pressing them individually onto a strip of plasticine kept in a covered petri dish. The needles were loaded while the first batch of embryos was accumulating. A clean needle was fixed onto the needle holder, connected to the oil line of a micro manipulator (Leitz). The tip of the needle was broken gently, by touching it to the end of a coverslip. Oil was forced into the needle until two-thirds full, (by turning the syringe of the micromanipulator), thus leaving space at the broken tip for loading DNA. The needle was then placed in a second needle holder connected to the vacuum line. A drop of DNA solution was placed onto a siliconized slide and the tip of the needle was immersed into it. Slight suction was applied so as to draw the DNA on the slide into the needle. When sufficient DNA was drawn into the needle, suction was stopped and the needle removed from the holder. All the air was expelled from the first needle holder connected to the oil

line and the needle loaded with the DNA was fxed onto it. While not in use, the tip of the needle was immersed in halocarbon oil, and placed on a slide.

(v). Dechorionation, mounting and desiccation of embryos

Initially, the embryos were chemically dechorionated using Chlorox liquid bleach. Later, only mechanical dechorionation was done under high humidity conditions. Three room humidifiers were used to keep the atmospheric humidity high. The embryos were first washed with distilled water to wash off the excess yeast paste and transferred to a 2.5 cm glass fibre filter. A pointed needle was used to lift the embryos off the filter and onto the double-sided sticky tape on the slide. The chorion was removed by rolling the embryos on the tape and the embryos were then lined up on the side of the sticky tape with the posterior ends pointing outwards. The dechorionated embryos were desiccated for 25-30 min. by placing the slides in petri dishes containing Drierite.

(vi)Injections

The desiccated embryos were covered with halocarbon oil to prevent further desiccation. The embryos of 0-4 hrs age were selected and injected with approximately 25 picoliters of DNA solution. To do this, the tip of the needle was gently pushed through the posterior end of the embryo and drawn back until it was right at the inside tip of the embryo. The syringe of the micromanipulator was gently turned to apply pressure which would force the DNA out of the needle. When a sufficient volume of DNA was injected, the needle was drawn out of the embryo while turning the syringe to remove the pressure applied.

(vii) Post-injection care:

The injected embryos were kept at 18° C until they hatched. The larvae were gently lifted off the oil and kept in a fly food plate containing yeast paste. When they became third instar larvae, they were transferred to standard fly food vials. The G_o flies were independently crossed to the parental genotype, and the G_1 flies were scored for the transformant phenotype.

22. Alcohol Dehydrogenase assays

(i) Spot tests

The procedure followed was as desribed in Grell et al. (1968). One to five flies were crushed onto Whatman 3 MM filter paper squares kept in a multi-welled, microtiter dish, in 100 µl of Tris-phosphate (pH 8.6). A 250 µl aliquot of staining mixture was then added and incubated at 22° C for 30 min in the dark . The staining mixture consisted of 90 ml of 0.05 M Tris-phosphate (pH 8.6), 4 ml NAD (10 mg/ml), 4 ml phenazine methosulfate (0.2 mg/ml), 2 ml nitroblue tetrazolium (NBT) (10 mg/ml) and 0.75 ml 2-butanol. A deep blue color indicates ADH activity.

(ii) Spectrophotometric assay

A Perkin-Elmer model 559A spectrophotometer was used for this purpose and the assays were done at 22° C. The ADH activity was measured by monitoring the rate of NAD reduction at 340 nm in the presence of 2-butanol as substrate. The assay mixture consisted of 0.1 ml of crude extract, and 0.9 ml of a solution containing 0.01 M sodium phosphate (pH 7.5), 0.14 M NaCl, 0.1 M 2-butanol, and 1.4 mM NAD^+. Crude extracts were made by homogenizing 60 mg of flies in 1 ml PBS (phosphate buffered saline). The homogenate was centrifuged at 14,000 rpm for 10 min. and the supernatant transferred to fresh Eppendorf tubes. Typically, the absorbance change was monitored for 10 min. and an average value was determined, and converted to number of units of ADH activity per minute. For conversion purposes, one unit of ADH activity was defined as a change of absorbance of 0.001 units per minute (Shermoen et al.,1987; Spencer, 1987).

23. Genomic DNA preparation from Drosophila

Genomic DNAs were prepared by following the method of Ish-Horowicz et al. (1979), with some modifications. A 400 mg sample of adult flies was homogenized in 6 ml of solution-I (10 mM Tris-Cl, pH 7.5, 60 mM EDTA, 0.15 mM spermidine, 0.15 mM

spermine, 0.2 mg/ml pronase E), and then 6 ml of solution II (0.2 M Tris-Cl, pH 9.0, 30 mM EDTA, 2% SDS, 0.2 mg/ml pronase E) were added, and the homogenate incubated at 37^0 C for one hour, with intermittent mixing. The homogenate was then extracted twice with buffer-saturated phenol, once with phenol:chloroform;IAA (50:48:2), and once with chloroform:IAA (24:1). NaCl was added to the final aqueous phase until the salt concentration was 0.2 M, followed by two volumes of 95% ethanol. The DNA was pelleted by centrifuging for 30 sec. at 1600x g. The pellet was washed once in 70% ethanol, dissolved in 4 ml TE and kept at 4° C overnight. RNase was then added to 25 µg/ml and incubated at 37° C for 30 min., followed by another ethanol precipitation. The DNA pellet obtained was dissolved in 1 ml of TE (pH 8.0). The DNA was then precipitated with spermine by adding KCl to 100 mM, and spermine to 10 mM, followed by centrifuging in a microfuge for 15 seconds. The supernatant was removed using a pasteur pipette, 1 ml of solution III (75% ethanol, 300 mM sodium acetate, 10 mM magnesium acetate) added, kept on ice for 2-4 hrs, and centrifuged again for 15 seconds. The supernatant was removed, again 1 ml of solution-III added, and the tube left overnight on ice. The pellet, after centrifugation as above, was washed twice with 70% ethanol, dried, redissolved in 100 µl TE (pH 8.0) and stored at 4° C.

24. Southern hybridizations of randomly primed probes to genomic DNA of Drosophila

Five µg of genomic DNA were digested with the appropriate restriction endonuclease(s) as described in section 9, and were electrophoresed on a 1.0% agarose gel (Ultrapure, BRL) gel for 12-16 hrs at 36 volts, with buffer circulation. The gel was stained in 100 ml of of ethidium bromide solution (0.5 µg/ml in distilled water) for 10 min. and photographed alongside a ruler. The DNA in the gel was then denatured for one hour in 150 ml of 0.5 N NaOH, 1.5 M NaCl and neutralized for one hour in 150 ml of 0.5 M Tris (pH 8.0), 1.5 M NaCl. Capillary transfer of DNA to Genescreen Plus membrane

(NEN Research Products) was set up according to the supplier's instructions. After the transfer, the membrane was air-dried for 2 hours and kept sealed in a plastic bag until used. The membrane was first prehybridized (Klessig and Berry, 1983) at 42° C for 2 hours in 50 ml of a solution containing 50% formamide, 500 µg/ml salmon sperm DNA, 1 M NaCl, 50 mM PIPES, pH 7.0, 0.5% Sarcosyl, 5x Denhardt's reagent, and 10 mM EDTA (pH 8.0), with constant and gentle shaking. The prehybridization buffer was then replaced with 10-15 ml of hybridization buffer (prehybridization buffer containing 10% dextran sulfate) and the appropriate α^{32}P-labeled probe (at a concentration of $1x10^6$ cpm/ml). Hybridization was continued for 12-16 hours at 42° C, with constant shaking, followed by washing as in the Genescreen Plus protocol. The membrane was then exposed to a Kodak XAR-5 film at -45° C for the required length of time.

25. Southern hybridizations of genomic DNA of Drosophila to end-labeled oligo-nucleotides

Digestions, electrophoresis, denaturation and transfer of genomic DNA were all done as described in section 24, except that 10 µg of DNA were used instead of 5 µg. The membrane was prehybridized and hybridized according to the method of van Tol et al. (1987) with some modifications. Prehybridization was done at 42° C for 5 hours in 10 ml of a solution containing 5x SSC, 5x Denhardt's, 1 mM EDTA, 0.01 M phosphate buffer pH 6.8, 1% SDS, 1 mM ATP, and 150 µg/ml of denatured salmon sperm DNA, followed by the addition of 20 picomoles of end-labeled oligo-nucleotide probe. Hybridization was continued for 12-16 hours at 42° C. The membrane was then washed three times at room temperature in 6x SSC, 5 min. each time, followed by one wash at 42° C in 6x SSC. The membrane was then exposed to a Kodak XAR-5 film for the required length of time before developing.

26. *In situ* hybridizations to salivary gland preparations of Drosophila

In situ hybridizations were done using the method of Pardue and Gall (1975), as modified by Glew et al. (1986). Regular cytology slides were prepared by dipping in a solution of 0.1% gelatin in distilled water at 65° C and air-drying for 2 hrs to overnight. The salivary glands were dissected in 45% acetic acid and transferred to a drop of 45% acetic acid on a gelatinized slide and covered with a #2 coverslip. Holding the coverslip at one edge, the glands were squashed, avoiding any lateral movement of coverslip. The slides were then dipped in liquid nitrogen, the coverslip lifted off with a razor blade, and the slide dipped once in 3:1 ethanol:acetic acid for 30-60 sec., twice in 95% ethanol for 5 min. each time, air-dried, and stored at 4° C until a sufficient number of slides were done to this stage. When ready for use, the slides were treated once with 2x SSC for 30 min. at 65-70° C, followed by twice with 70% ethanol, and twice with 95% ethanol, five min. each time at room temperature. The slides were then air-dried and the DNA was denatured by dipping once in 0.07 N NaOH for 3 min., followed by three times in 70% ethanol, and twice in 95% ethanol, five min. each time at room temperature. For the hybridization reaction, nick-translated probe and denatured salmon sperm DNA (20 mg/ml) were mixed in equal volumes and kept in a boiling water bath for 3 to 5 min. They were then mixed with an equal volume of hybridization buffer (4x Denhardt's, 20% dextran sulfate, 1.3 M NaCl, 0.04 M $MgCl_2$ and 0.04 M sodium phosphate, pH 7.0). Twenty µl of the above solution were placed on each slide, covered with a coverslip, and placed in a slide box containing Kimwipes soaked in 2x SSC. The box was sealed and kept in a 65° C waterbath overnight. After the hybridization, the slides were washed three times in 2x SSC at 65° C for 15 min. each time, once in 70% ethanol and twice in 95% ethanol at room temperature, 5 min each time. The slides were then air-dried. The slides were then coated with Kodak NTB-2 Nuclear Track emulsion (prepared according to manufacturer's specifications), by dipping the slides at 60° C, and air-drying. The slides were then placed

in a slide box containing some Drierite, sealed with black tape, and kept at 4° C until developed. The slides were developed at room temperature using Kodak Dektol developer and Kodak fixer prepared according to supplier's specifications. The slides were placed for one minute in distilled water, 2.5 min. in the developer, 0.5 min. in distilled water, 4-5 min. in fixer and 5 min. in distilled water. The slides were finally rinsed in distilled water for 15 min. before staining with Giemsa. The stain was prepared by adding 0.5 g of Giemsa to 35 ml of glycerol, heating to 60° C for 2 hours, with stirring, followed by addition of 33 ml of absolute methanol. A 5-10 ml aliquot of the Giemsa stock solution was diluted in 100 ml of 10 mM sodium phosphate, pH 7.0. The slides were dipped into this diluted stain, followed by rinsing in distilled water to the desired degree of staining, and air-drying. One drop of Permount (Fisher Scientific) was added to the slides, a coverslip placed on top, and the slides were stored at 4° C.

27. Preparation of tRNAs from yeast

The method used was as described in Lo et al. (1982). Yeast strains of the appropriate genotype were grown to stationary phase in the appropriate medium at 30° C, with vigorous shaking. The cells were pelleted at 5000 rpm., and resuspended in extraction buffer (0.05 M Tris-HCl, pH 7.5, 0.05 M $MgCl_2$ and 0.01 M β-mercaptoethanol) in a volume of 1.5 ml of buffer/g wet weight. An equal volume of buffer-saturated phenol was added, stirred for approximately 12 hrs at 4° C, followed by centrifugation at 10,000 rpm for 45 min. at 4° C. The aqueous phase was transferred to a 30 ml Corex tube, and 0.1 volume of 4 M potassium acetate and 2.5 volumes of 95% ethanol were added and the tube kept at -20° C overnight. The RNA was pelleted by centrifuging at 10,000 rpm for 10 min. at 4° C and redissolved in 10 ml of starting buffer (0.05 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$, 0.001 M EDTA, and 0.01 β-mercaptoethanol). The tRNAs were then recovered after subsequent DEAE-cellulose column chromatography (Section 29).

28. Preparation of tRNAs from Drosophila

Total RNA was prepared by the method of Gietz and Hodgetts (1985), with some modifications. One gram of flies was homogenized in 10 ml of RNA extraction buffer (30 mM Tris-HCl, pH 7.5 at 4° C, 100 mM NaCl, 5 mM KCl, 10 mM Mg SO_4, 5 mM DTT, 4 mM EGTA, 5 mM N-ethyl maleimide, 0.5% (v/v) β-ME, 0.5% (v/v) Nonidet P-40, 25 µg/ml polyvinyl sulfate, 35 µg/ml spermine). The homogenate was transferred to a 30 ml Corex tube and SDS added to 1% (w/v) followed by two extractions with buffer-saturated phenol:chlorofrom:IAA (25:24:1), and two extractions with chloroform:IAA (24:1). The RNA from the final aqueous phase was precipitated with 2.5 vol. of 95% ethanol at -20° C overnight. The RNA was pelleted by centrifuging at 10,000 rpm for 10 min., dried, redissolved in 10 ml of starting buffer (section 27), and the tRNAs collected after DEAE cellulose column chromatography (Section 29).

29. DEAE-cellulose column chromatography

An 8 ml column of DEAE-cellulose was poured in a 10 ml syringe plugged with glass wool. The column was always kept from drying by keeping starting buffer (section 27) on top. After equilibration with 40 ml of starting buffer, the crude RNA, dissolved in starting buffer, was loaded onto the column. The nucleoside polyphosphates were eluted using a low salt buffer (0.25 M NaCl in starting buffer). The tRNAs and other smaller RNAs were then eluted using a higher salt buffer (1.0 M NaCl in starting buffer). To precipitate the tRNA, 0.1 volume of 4 M potassium acetate (pH 4.5) and 2.5 volumes of ethanol were added to the eluate. The tube was kept overnight at -20° C, followed by centrifugation at 10,000 rpm for 10 min. The pellet was dried and dissolved in 0.5-1.0 ml of tRNA storage buffer (0.05 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$, 0.001 M EDTA). The concentration of tRNA was determined from A_{260} values, and stored at -20° C.

30. Northern hybridizations to tRNA extracted from yeast

The procedure used was that of van Tol et al. (1987). The gels used for the separation of RNAs were 10% polyacrylamide and 8.3 M urea in 1x TBE. The gels were pre-electrophoresed in 1x TBE (pH 8.3) for 30 min. at 110 volts. Thirty µg of each tRNA preparation were dried in a Speed-vac drier, dissolved in 7.0 µl of formamide dye (described in section 14), loaded onto the gel, and electrophoresed at 290 volts until the bromophenol blue dye ran off the gel. The gel was stained for 10 min. in 100 ml of ethidium bromide solution (0.5 µg/ml of distilled water) and photographed alongside a ruler. The transfer to Genescreen Plus membrane was set up using Bio-Rad's Transblot apparatus following the manufacturer's instructions. The transfer was made in 1x TAE for 12 hours at 0.18 Amp constant current at 4° C, with gentle stirring of the buffer. The membrane was briefly rinsed in 1x TAE after transfer, air-dried for 2 hrs and kept sealed in a bag until use. During prehybridization, the membrane was placed in a plastic bag with 10 ml of the prehybridization solution (described in section 25) and incubated at 42° C, with gentle shaking, for 5 hours. Appropriately end-labeled oligonucleotide probes (10 picomoles, described in section 19) were then added to the same solution, the bag resealed, and hybridized at 42° C for 12-16 hours, with constant shaking. After hybridization, the membrane was washed 3 times in 6x SSC at room temperature, 10 min. each time, followed by one wash at 57° C for 30 min. The membrane was then wrapped in Saran Wrap and autoradiographed.

31. Northern hybridizations to tRNAs extracted from flies

The preparation of gels, electrophoresis and blotting of tRNAs to Gene Screen Plus membrane were done as described in section 30. Prehybridizations and hybridizations were done in the buffer described in section 25 except that 30° C was used instead of 42° C. The washing was done 3 times in 6x SSC, 5 min. each time at room temperature followed by a two minute wash at 39° C (Conner et al., 1983), and autoradiographed.

32. Immunoprecipitations and competition assays

Crude extracts of flies were made in PBS buffer (0.01 M sodium phosphate pH 7.5 and 0.14 M NaCl) as described in section 22. Immunoprecipitations were done using the method of W. Sofer (personal communication). A constant volume (50 µl) (Section 22.ii) of crude extracts was mixed with different volumes of goat anti-ADH antiserum in a final volume of 500 µl. The tubes were incubated at 4° C overnight and centrifuged for 10 min. at 13,000 rpm at 4° C. The supernatants were transferred to fresh Eppendorf tubes and assayed for the ADH activity remaining in the supernatant as described in section 22. ii. To study the effect of competition by the antigen for the antibodies, two extracts were mixed, immunoprecipitated as above, and assayed for the ADH activity remaining in the supernatant as described in section 22. ii.

33. Autofluorography

The nitrocellulose filters or the Genescreen Plus membranes used in hybridization to all radio-labeled probes were washed as required and exposed to a Kodak XAR-5 film in a Kodak X-Ray cassette at -45° C, with two intensifying screens. After exposure for the required length of time, the film was developed using Kodak X-Ray film developer and fixer following the manufacturer's instructions.

Chapter III

RESULTS

As outlined in the Introduction, this project was initiated with the objective of constructing a repertoire of biologically active nonsense suppressor alleles of a $tRNA^{Ser}$ gene of *Drosophila melanogaster*.

1. Choice of the tRNA gene

The choice of the tRNA gene was based on the finding that in yeast all of the natural nonsense suppressors involve altered forms of tyrosine, leucine or serine tRNAs. Since tyrosine and leucine tRNAs of Drosophila were already being studied by other groups (Dr. E. Kubli in Switzerland and Dr. D. Hartl in St. Louis, respectively), our laboratory focussed on serine tRNA of *D. melanogaster*. When the project was started, the only nonsense alleles known in Drosophila (Adh^{nB}, Kubli et al., 1982; and $lfm(3)^7$ allele of the Act 88F gene, Karlik et al., 1984) were both tryptophan (UGG) to opal (UGA) mutations. In the Adh^{nB} mutation, the tryptophan codon at position 234 (Benyajati et al., 1981) is changed to an opal codon (Kubli et al., 1982). In the case of the $lfm\ (3)^7$ mutation (Mogami and Hotta, 1981), the tryptophan codon at position 355 of the Act 88F actin gene is changed to an opal nonsense codon by a G to A transition (Karlik et al.,1984). Since the gene coding for the $tRNA^{Trp}$ in Drosophila was not yet isolated, and since many of the nonsense mutations in yeast were found to be suppressible by serine-inserting suppressors (Ono et al., 1979; Olson et al., 1981; Krupp et al., 1985), a gene coding for $tRNA^{Ser}$ was thought to be a logical choice for this work.

2. The gene for $tRNA^{Ser}_7$ of *D. melanogaster*

The gene coding for $tRNA^{Ser}_7$ (recognizing the UCN codon family) in Drosophila was used in this work. This gene was isolated and cloned by Dr. G.M. Tener's group

(Dunn et al., 1979). It is found on chromosome II (band 23E). A 4.4 kb *Hin*dIII fragment containing this tRNA gene was cloned into the *Hin*dIII site of the pBR322 plasmid (Dunn et al., 1979). The resulting plasmid was called pDt5. The sequence of the gene has been determined (Cribbs et al., 1987b). It is 82 base pairs in length with an AGA anticodon (modified to IGA in the tRNA). The sequence of the tRNA gene as determined by Cribbs et al. (1987b) is represented in Fig. 3, and the corresponding cloverleaf structure of the tRNA (Cribbs et al., 1987a) in Fig. 4. A partial restriction map of the 4.4 kb *Hin*dIII fragment, as modified and improved during this work, is presented in Fig. 5.

3. Sub-cloning into M13 mp18

A 2.4 kb *Pst*I-*Hin*dIII DNA fragment from pDt5, containing this $tRNA_7^{Ser}$ gene (Fig. 5), was sub-cloned into the M13 mp18 phage vector, as described in Materials and Methods. The sequence of the tRNA gene within this phage recombinant, M-PH1 (Fig. 5) was confirmed by the dideoxy chain termination method (Sanger et al., 1977), using the oligonucleotide 'a14' (Fig. 6) as the primer for sequencing. The sequence was found to be in agreement with the sequence as presented in Fig. 3. Single-stranded DNA templates of this construct were used in all the mutagenesis experiments described below.

4. Construction of the nonsense suppressor mutant alleles of the $tRNA^{Ser}$ gene of *D. melanogaster*

Oligonucleotide-directed, site specific mutagenesis (Zoller and Smith, 1982; Kunkel, 1985; Stewart et al., 1985) was used to construct the nonsense suppressor tRNA genes used in this work. Templates containing deoxyuridine substitutions (Kunkel, 1985) were used in all the mutagenesis experiments and the strategy utilized in this study is schematically represented in Fig. 7. M-PH1 phage were grown in the strain BW313 (*dut ung*; deficient in both dUTPase and uracil N-glycosylase), in the presence of deoxyuridine and single stranded DNA templates were generated. The resulting templates contained

Fig. 3. The nucleotide sequence of the region of M-PH1, containing the $tRNA^{Ser}$ gene from band 23E of *Drosophila melanogaster* (adapted from Cribbs et al., 1987b). The orientation of the tRNA gene is indicated by an arrow. The boxes indicate the coding region of the tRNA gene. The nucleotide that corresponds to the 5' terminal nucleotide of the mature tRNA has been designated number 1. All the nucleotides 3' to this reference point were given positive values and those 5' to this nucleotide were given negative values. The coding strand is the bottom strand, and the anticodon region is denoted by bold face letters.

-70 -60 -50 -40 -30 -20
GGTTCCGAAATGTGTGTTCAGTCTTGGATTCTCCCATGCAATCAACATTAGTTGCCAATT
CCAAGGCTTTACACACAAGTCAGAACCTAAGAGGGTACGTTAGTTGTAATCAACGGTTAA
-10 1 10 20 30 40
TGCCGTGTCATATTAACAGCAGTCGTGGCCGAGCGGTTAAGGCGTCTGACTAGAAATCAGA
ACGGCACAGTATAATTGTCGTCAGCACCGGCTCGCCAATTCCGCAGACTGATCTTTAGTCT
50 60 70 80 90 100
TTCCCTCTGGGAGCGTAGGTTCGAATCCTACCGACTGCGAGAAGGTTTACGGATTTTTTTT
AAGGGAGACCCTCGCATCCAAGCTTAGGATGGCTGACGCTCTTCCAAATGCCTAAAAAAAA
110 120 130 140 150 160
ATTTTTCAACATTCTCTATTCTATTCTCTATTTTTTCCTCTCAGAAACTGGTTAAATTATT
TAAAAAGTTGTAAGAGATAAGATAAGAGATAAAAAAAGGAGAGTCTTTGACCAATTTAATAA
170 180 190 200
ATAATAATAATACATACATGTGTGCTGTTTGTTTACAACT
TATTATTATTATGTATGTACACACGACAAACAAATGTTGA

Fig. 4. Cloverleaf structure of the $tRNA^{Ser}_{1}$ of *D. melanogaster* (from Cribbs et al., 1987a). The sequence of the gene corresponding to the anticodon IGA is AGA. The 3' terminal CCA sequence is added post transcriptionally.

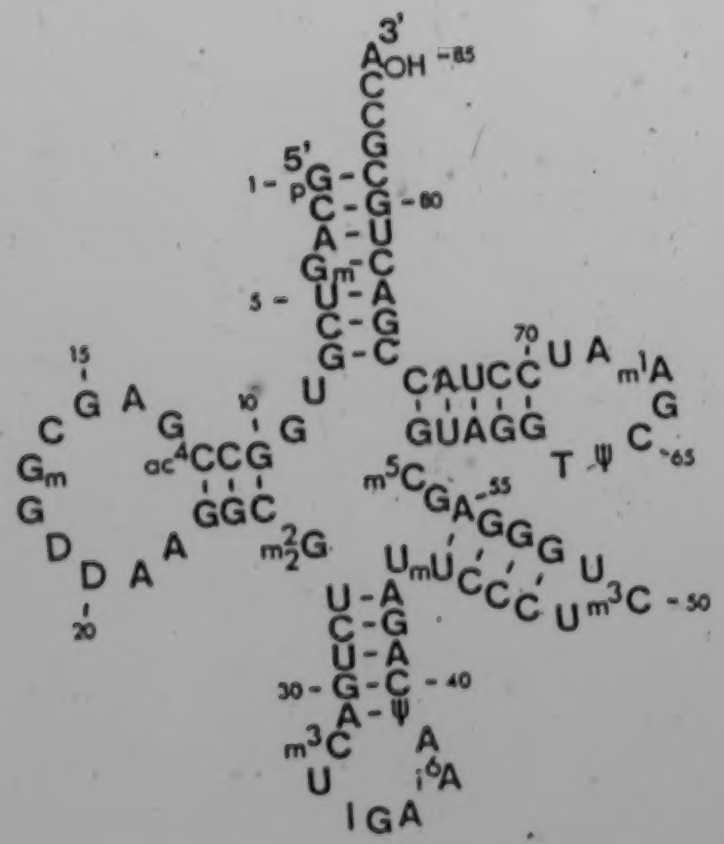

3'
OH
5'
pG
m7G
ac4C
m22G
m5C
m1A
m3C
Ψ
i6A
I

Fig. 5. Restriction map of the 4.4 kb *Hin*dIII fragment containing the $tRNA_7^{Ser}$ gene of *D. melanogaster* (adapted and improved from Dunn et al., 1979), and the strategy used to clone the $tRNA_7^{Ser}$ wild type gene into the M13mp18 phage vector.

The 2.4 kb *Pst*1-*Hin*dIII fragment was purified from a LMP agarose gel as described in Materials and Methods and ligated into the *Pst*1-*Hin*dIII site in the cloning cassette of appropriately restricted M13mp18 (Maniatis et al., 1982), followed by transformation of the *E. coli* host strain, JM105. A colorless plaque was selected, characterized, and used for further studies. The wide bar at the top of the figure represents the 4.4 kb *Hin*dIII *D. melanogaster* DNA fragment, and the small arrow indicates the approximate location of the $tRNA_7^{Ser}$ gene.

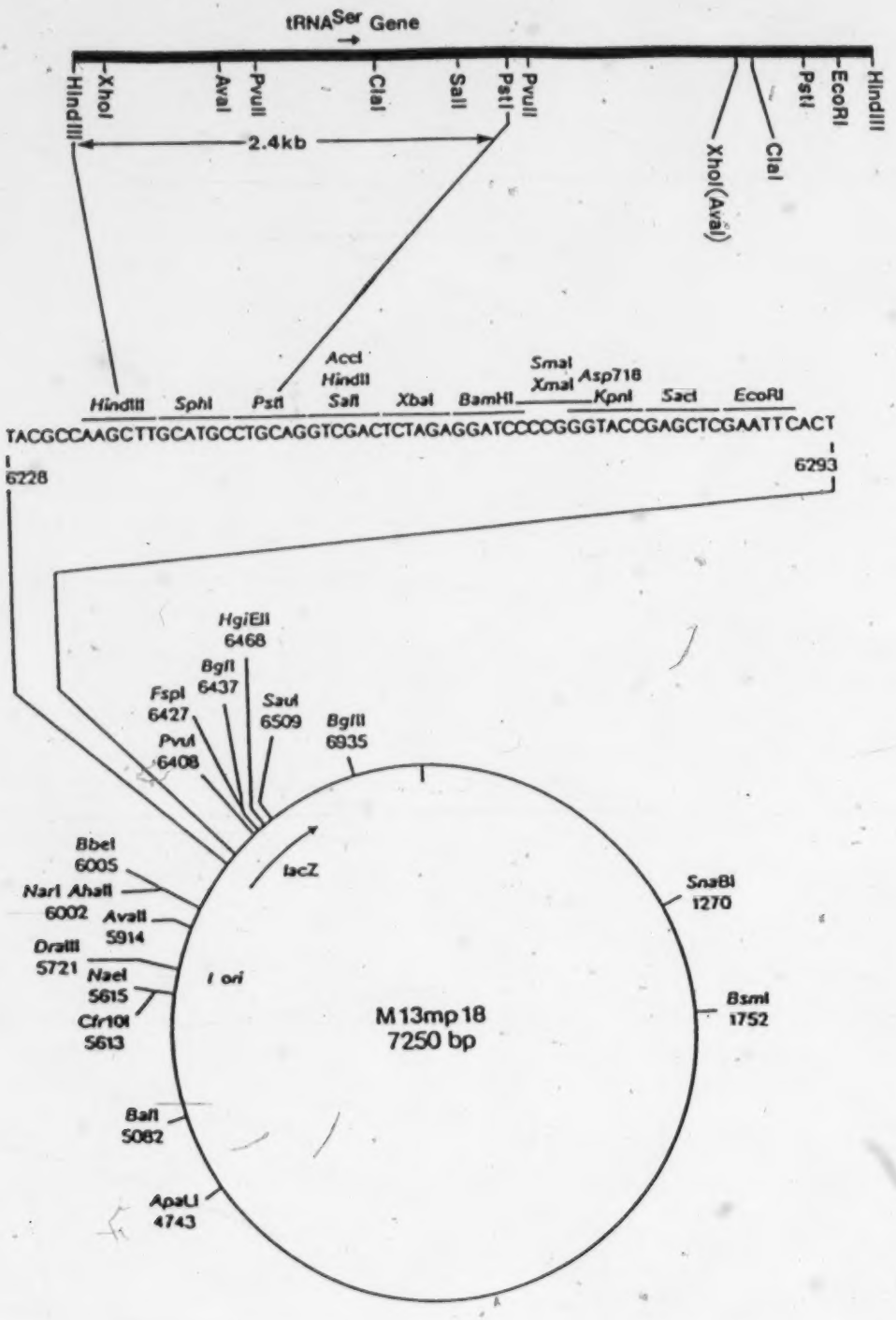

tRNA^Ser Gene
HindIII
XhoI
AvaI
PvuII
ClaI
SalI
PstI
PvuII
XhoI(AvaI)
ClaI
PstI
EcoRI
HindIII
2.4kb
AccI
HindII
SalI
SmaI
XmaI
Asp718
KpnI
HindIII
SphI
PstI
XbaI
BamHI
SacI
EcoRI
TACGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCACT
6228
6293
HgiEII
6468
BglI
6437
FspI
6427
PvuI
6408
SauI
6509
BglII
6935
BbeI
6005
NarI AhaII
6002
AvaII
5914
DraIII
5721
NaeI
5615
Cfr10I
5613
lacZ
f ori
M13mp18
7250 bp
SnaBI
1270
BsmI
1752
BalI
5082
ApaLI
4743

Fig. 6 Oligonucleotides used as the primers for the sequencing and *in vitro* mutagenesis experiments, and as probes in the Southern and Northern hybridization analyses. Oligonucleotides represented above the wild type sequence indicate that they have the same sequence as the top strand of the wild type sequence and the ones below indicate that they have the same sequence as the bottom strand. For all oligonucleotides, only the bases which differ from the wild type are indicated. The arrow in the figure indicates the direction of transcription. The nucleotide that corresponds to the 5' terminal nucleotide of the mature tRNA has been designated number 1. All the nucleotides 3' to this reference point were given positive values and those 5' to this nucleotide were given negative values. The coding strand is the bottom strand in the figure. **a14**: A 14 base oligonucleotide primer used in sequencing the tRNA genes when in M13 or plasmid derivatives. It was also used as the upstream primer in all the *in vitro* mutagenesis experiments. **op16, oc16, and am16**: mutant oligonucleotides (each 16 bases long) used in the construction of the opal, ochre and amber mutant alleles, respectively, of the $tRNA^{Ser}$ gene of *D. melanogaster*. The above oligomers were obtained from the DNA synthesis laboratory, University of Calgary. **SPA#1**: used as a probe in the Northern analyses presented in Figs. 11, 12, 28, and 30; and in the Southern hybridization analysis presented in Fig. 23. **SPA#2 and 5**: used as cold competitors for the wild type $tRNA^{Ser}$ (IGA & CGA) of *D. melanogaster* in the Northern analyses presented in Figs. 27 and 29. **SPA#3 and 4**: primers that were complementary to the anticodon region of the opal and amber suppressor $tRNA^{Ser}$ of Drosophila which were used as end-labeled probes in the Northern analyses presented in Figs. 27 and 29, respectively. The oligonucleotides designated SPA#1 to SPA#5 were kindly provided by Dr. K.L. Roy.

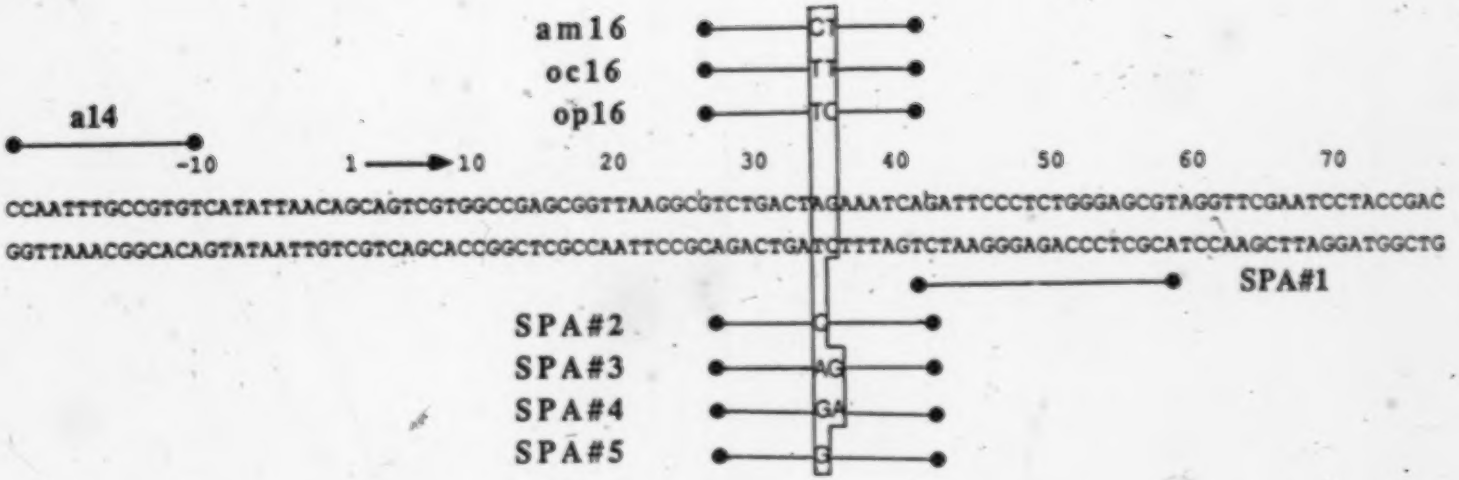
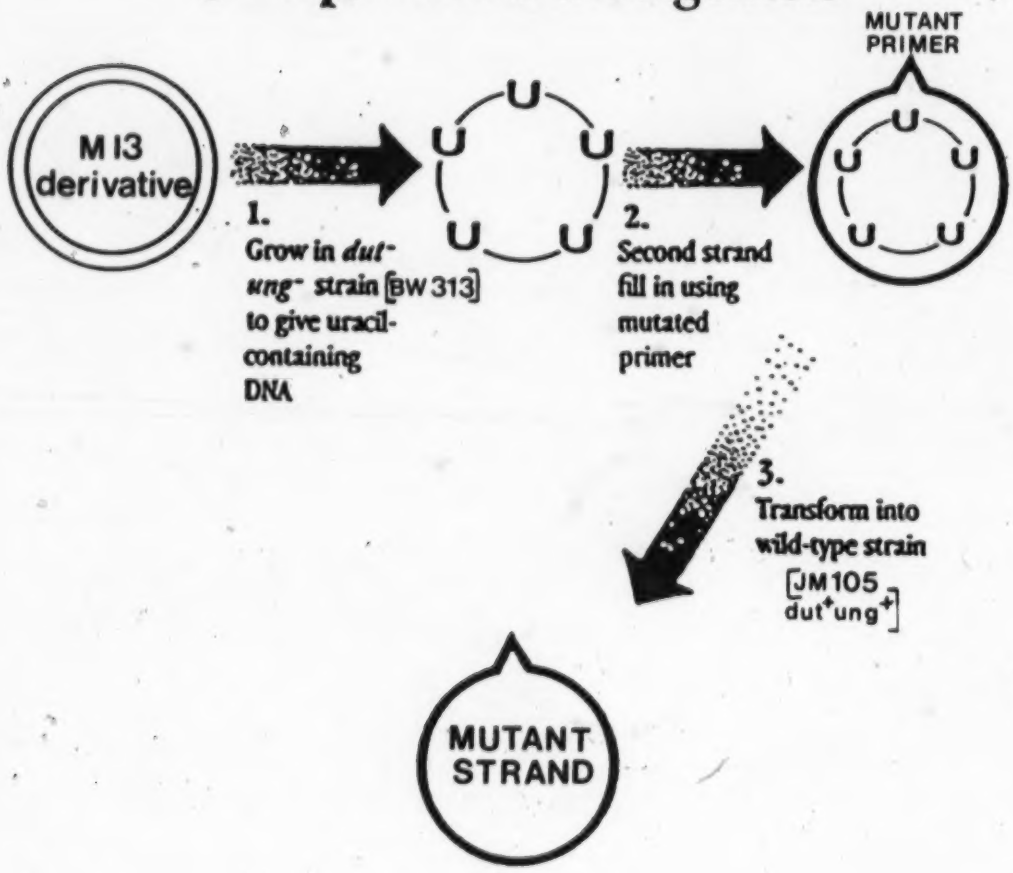

am16
oc16
op16
a14
-10
1
10
20
30
40
50
60
70
CCAATTTGCCGTGTCATATTAACAGCAGTCGTGGCCGAGCGGTTAAGGCGTCTGACTAGAAATCAGATTCCCTCTGGGAGCGTAGGTTCGAATCCTACCGAC
GGTTAAACGGCACAGTATAATTGTCGTCAGCACCGGCTCGCCAATTCCGCAGACTGATCTTTAGTCTAAGGGAGACCCTCGCATCCAAGCTTAGGATGGCTG
SPA#1
SPA#2
SPA#3
SPA#4
SPA#5

Fig. 7 Schematic diagram of the strategy used in the oligonucleotide-directed, site-specific mutagenesis. (from Bio-Rad laboratories, Bulletin #1409).

3-Step *in vitro* mutagenesis


MUTANT PRIMER
M13 derivative
1. Grow in *dut⁻ ung⁻* strain [BW 313] to give uracil-containing DNA
2. Second strand fill in using mutated primer
3. Transform into wild-type strain [JM105 dut⁺ung⁺]
MUTANT STRAND

uracil incorporated at a fraction of positions normally containing thymine. The mutant primer was then annealed to these templates, and the second strand synthesized in the absence of uracil, as described in section 16 of Materials and Methods. Oligonucleotides used in the mutagenesis, and in the Northern and Southern hybridizations are represented in Fig. 6. In addition to the mutant oligonucleotide, another oligomer that anneals upstream (from -11 to -24) of the start of the tRNA gene coding region was also used in the mutagenesis reaction. This double primer method is reported to prevent displacement of mutant oligonucleotides from the template and increase the efficiency of recovery of mutants (Norris et al.,1983). After the second strand synthesis, strain JM105 was transformed with this construct. In this strain, the parental strand is selected against because JM105 contains dUTPase and uracil N-glycosylase. This leads to an increased recovery of the desired mutants. Plaques were screened and the mutants identified by plaque hybridization (Benton and Davis, 1977) to the appropriate mutant oligonucleotide (Fig. 6). The base changes in the gene were confirmed by dideoxy chain termination DNA sequencing (Fig. 8). Each of the desired sequence changes, for the three suppressor constructs (opal, ochre and amber) made, required two nucleotide substitutions from the wild type $tRNA^{Ser}$ gene. These changes are represented diagrammatically in Fig. 9. The entire gene sequence was checked in each case to verify that no other changes were introduced into the gene. The recovery of mutants using this procedure was found to be about 10-20%, somewhat lower than theoretical expectations.

5. Sub-cloning into YRp7 vector

To study the expression of these engineered Drosophila tRNA genes in a heterologous system, the mutant constructs were subcloned into a yeast (*S. cerevisiae*) replicating plasmid, YRp7. YRp7 is an *E. coli*-yeast shuttle vector (Tschumper and Carbon, 1980) with ampicillin resistance as the selectable marker in *E. coli* and the wild type sequence for the *TRP*1 gene for selection in *S. cerevisiae*. A 2.1 kb *Sal*I-*Xho*I

Fig. 8 Autoradiographs of 6% polyacrylamide, 8.3 M urea sequencing gels representing the anticodon region of the wild type $tRNA^{Ser}$ gene and its nonsense suppressor derivatives. In order to confirm the base changes introduced into the anticodon, the non-coding strand of the genes was sequenced, using the dideoxy chain termination procedure (Sanger et al., 1977).

The seqences represent, from left to right, the wild type gene, followed by its opal, ochre and amber derivatives respectively. The anticodon regions of the genes are indicated by the lettered sequences (AGA for wild type and so on) on the right side of each picture.

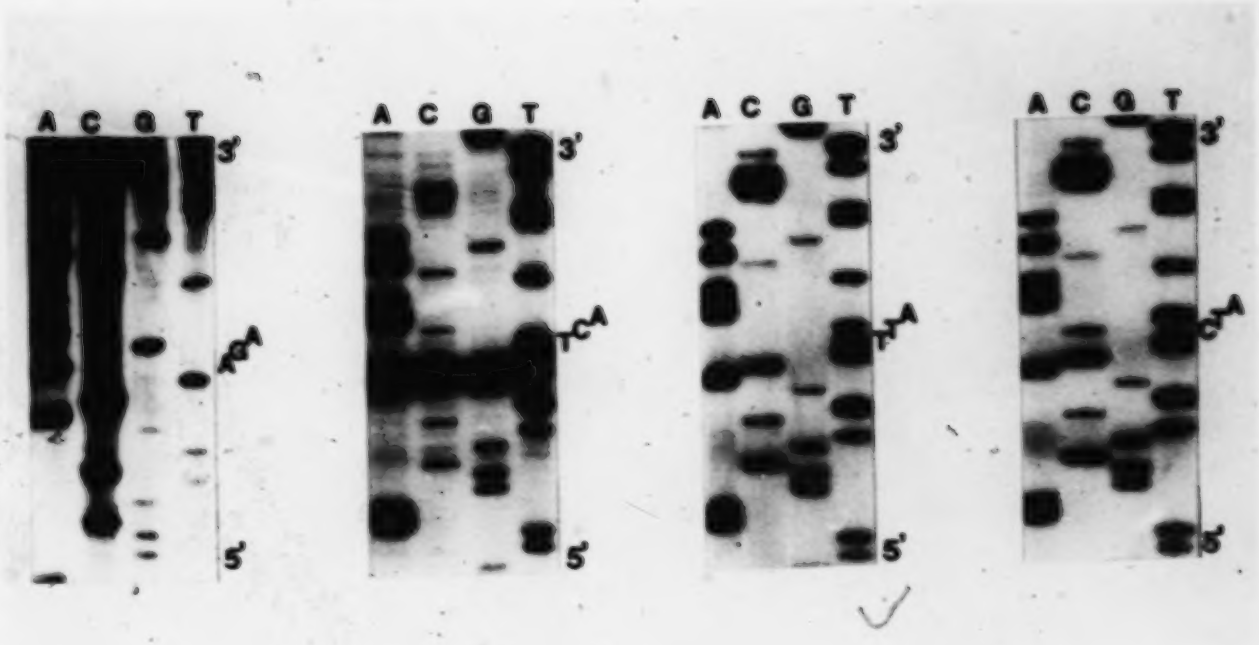
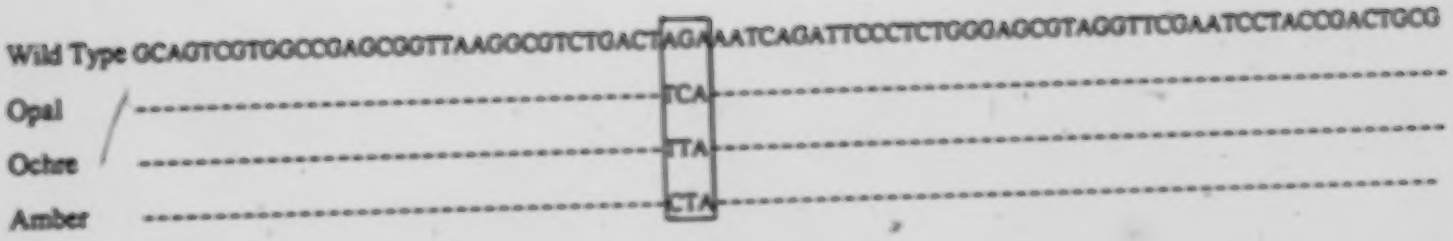

A C G T
3'
5'
A C G T
3'
5'
A C G T
3'
5'
A C G T
3'
5'

Fig. 9 Nucleotide sequences of the genes for $tRNA^{Ser}$ and the derivatives constructed by site-specific mutagenesis. The complete sequence of the wild type gene is represented in the top line. For the mutant derivatives, only the changes made in the wild type anticodon are represented below the corresponding wild type bases and the anticodon regions of all the constructs are boxed.


Wild Type GCAGTCGTGGCCGAGCGGTTAAGGCGTCTGACTAGAAATCAGATTCCCTCTGGGAGCGTAGGTTCGAATCCTACCGACTGCG
Opal
TCA
Ochre
TTA
Amber
CTA

fragment (Fig. 5), containing the wild type tRNA^{Ser} gene or one of its mutant derivatives, was subcloned into the *Sal*I site of the YRp7 vector (Fig. 10). The positive clones in *E. coli* were identified by ampicillin resistance selection and subsequent colony hybridization to appropriate (Fig. 6) 5' end-labeled oligonucleotides and by restriction endonuclease mapping. The entire sequence of the tRNA gene was again confirmed for each construct using double stranded DNA sequencing (Chen and Seeburg, 1985).

6. Transformation of *S. cerevisiae* and *in vivo* suppression assays

The yeast strain used as the host in the transformation assays was JG 369-3B (α) (a gift from Dr. Jean Paul Gelugne) with an array of nonsense alleles (Table 3), which made it suitable for the analysis done. All the *in vitro* constructed nonsense suppressor tRNA genes and their wild type counterpart were individually introduced into the above yeast strain.

The host strain used in the transformations contains the *trp1-1* allele. Since the vector carried the wild type allele (*TRP*1) of this gene, the yeast transformants were first selected on tryptophan omission medium and later tested on media lacking tryptophan and the end product relevant to one of the nonsense alleles. Colonies representing individual transformants could be observed after 2-3 days of incubation at 30° C. As represented in Table 3, the suppressor derivatives of the tRNA^{Ser} gene of *D. melanogaster* were found to function in a codon-specific manner in the yeast *S. cerevisiae*. The only exceptions found were *ade*2-1 and *can*1-100. The former was poorly suppressed, and the latter was not suppressed at all by the Drosophila genes. Suppression of *TRP*1-1 was not assayed for since it was used as the selectable marker.

7. Expression of *D. melanogaster* tRNA^{Ser} genes in *S. cerevisiae*

The functional assays of the *D. melanogaster* tRNA^{Ser} gene constructs in *S. cerevisiae* indicated that these genes are biologically active in the latter though they are of

Fig. 10 Schematic diagram of the strategy used to clone the wild type and the nonsense suppressor derivatives of the $tRNA^{Ser}$ gene of *D.melanogaster* into the *E. coli*-yeast shuttle vector, YRp7. On the right is the 2.4 kb *Pst*I-*Hin*dIII *D.melanogaster* DNA fragment present in the M13 mp18 vector (containing either the wild type $tRNA^{Ser}_7$ gene or its mutant derivatives) from which, a 2.1 kb *Sal*I-*Xho*I fragment was cloned into the unique *Sal*I site in YRp7, as indicated. The YRp7 diagram is from Pouwels et al. (1988).

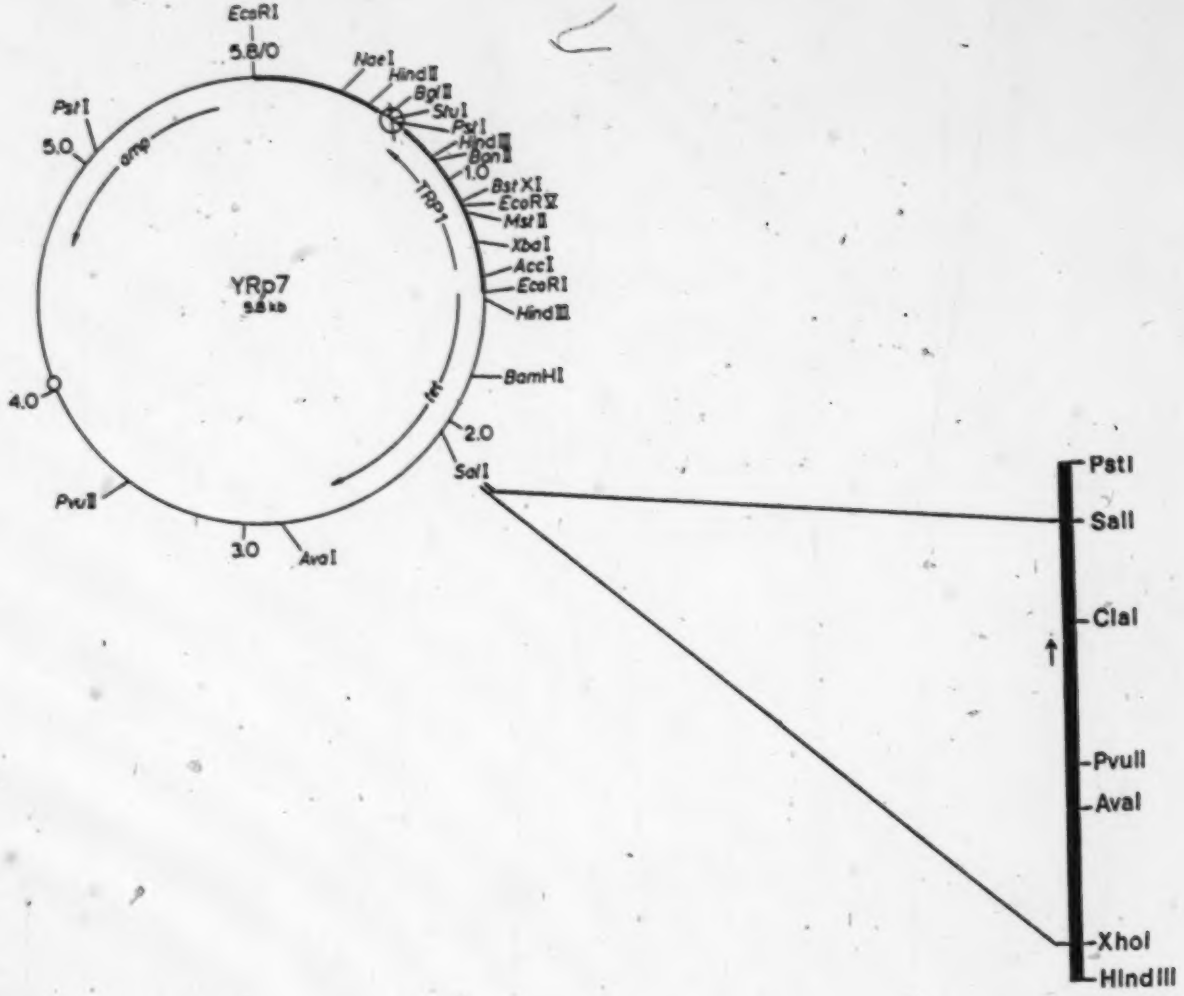

EcoRI
5.8/0
NaeI
HindII
BglII
StuI
PstI
HindIII
BglII
1.0
BstXI
EcoRV
MstII
XbaI
AccI
EcoRI
HindIII
BamHI
2.0
SalI
PstI
SalI
ClaI
PvuII
AvaI
XhoI
HindIII
PstI
5.0
amp
TRP1
YRp7
5.8 kb
ori
4.0
PvuII
3.0
AvaI

Table 3. *In vivo* assays of the *Drosophila* tRNA ^{Ser} wild type and mutant constructs in *S. cerevisiae*.

a. The genotype of the strain, JG 369-3B (α) used as the recipient in the above transformation assays is:
*ade*2-1, *lys*2-1, *can*1-100 (UAA alleles)
*met*8-1, *trp*1-1 (UAG alleles)
*leu*2-2, *his*4-260 (UGA alleles)
*ura*3-52 (non-suppressible, selectable marker)

b. The derivatives of YRp7 in the above table contain the wild type construct of the $tRNA^{Ser}_{IGA}$ gene (pSS166), or respectively the *in vitro* constructed $tRNA^{Ser}_{UCA}$ (pSS152), $tRNA^{Ser}_{UUA}$ (pSS179) or $tRNA^{Ser}_{CUA}$ (pSS182) genes. The codons suppressed by each of the constructs were indicated within the brackets.

c. (+), suppression of the nonsense allele
(-), no suppression
(+/-), low efficiency of suppression

Table 3. *In vivo* assays of the Drosophila $tRNA^{Ser}$ wild type and mutant gene constructs in *S. cerevisiae*.

TRANSFORMANT		SUPPRESSOR PHENOTYPE[a]					
		ade2-1 (UAA)	*lys2-1* (UAA)	*can1-100* (UAA)	*met8-1* (UAG)	*leu2-2* (UGA)	*his4-260* (UGA)
YRp7 [b]	----	-[c]	-	-	-	-	-
pSS166	----	-	-	-	-	-	-
pSS152	(UGA)	-	-	-	-	+	+
pSS179	(UAA)	+/-	+	-	-	-	-
pSS182	(UAG)	-	-	-	+	-	-

heterologous origin. To assess the levels of expression and the accuracy of processing of these Drosophila genes in the *S. cerevisiae*, Northern hybridization analysis was done on tRNAs fractionated from the total RNA from each transformant. The probe used was SPA#1 (Fig. 6), a 19 base oligonucleotide that is capable of hybridizing to the Drosophila $tRNA^{Ser}$ wild type and mutant tRNA sequences, since it is complementary to identical positions in all these genes. A comparison of all the published sequences coding for $tRNA^{Ser}$ from *S. cerevisiae* and Drosophila (Sprinzl et al., 1989) revealed that there is an approximate 68% overall homology between the yeast and Drosophila tRNA gene sequences. SPA#1 was chosen from a region of the Drosophila $tRNA^{Ser}$ gene sequence that has the least homology (33-47%) to the published yeast $tRNA^{Ser}$ gene sequences, thus minimizing the possibility of cross-hybridization to yeast tRNAs under the conditions used. As indicated in Fig. 11, all the Drosophila $tRNA^{Ser}$ genes were transcribed and processed well in the yeast, resulting in a tRNA species of the expected size. The absence of any signal in the lanes containing tRNA from the untransformed yeast strain, or from the yeast strain transformed with the vector alone, (YRp7) indicates that the signal in the other lanes specifically represents Drosophila tRNA species.

To further prove that these tRNA species were transcribed from the genes carried on the plasmid, another experiment was done. The transformant yeast strains were grown on complete medium to relax selection for retention of the plasmid, thus allowing the loss of the plasmid (ascertained on the basis of inability subsequently to grow on tryptophan omission medium). The concomitant loss of suppression was assayed by testing on appropriate omission media (Data not shown). The results further verify that the suppressor activity was tightly linked with the presence of the plasmid. The tRNAs were then purified from these strains and analyzed on a Northern blot. There was a complete loss of signal from all the lanes containing tRNA from yeasts which had lost the suppressor ability, as shown in Fig. 12.

Fig. 11 Autoradiograph of the Northern hybridization analysis of the tRNAs from the yeast transformant strains assaying for the presence of Drosophila tRNA species.
Lane 1, tRNA from an untransformed yeast strain, JG 369-3B (α);
Lane 2, *E.coli* tyrosine tRNA used as a size marker in the ethidium bromide stained gels.
Lanes 3-7, tRNA from the above yeast strain transformed with the plasmid vector YRp7 or the plasmid containing the wildtype gene for $tRNA^{Ser}_{UCN}$ (pSS166) or its opal (pSS152), ochre (pSS179) or amber (pSS182) suppressor derivatives respectively.

The isolation and purification of the yeast tRNA was done as described in Lo et al (1982). The tRNAs were run on a 10% acrylamide-8M urea gel and blotted onto a Gene Screen Plus membrane using a Bio-Rad Trans-Blot cell according to the manufacturer's recommendations. The oligonucleotide used as a specific probe for Drosophila transcripts is SPA#1 (Fig. 6). It was 5' end labeled using [γ-^{32}P]ATP (4500 Ci/mmol)

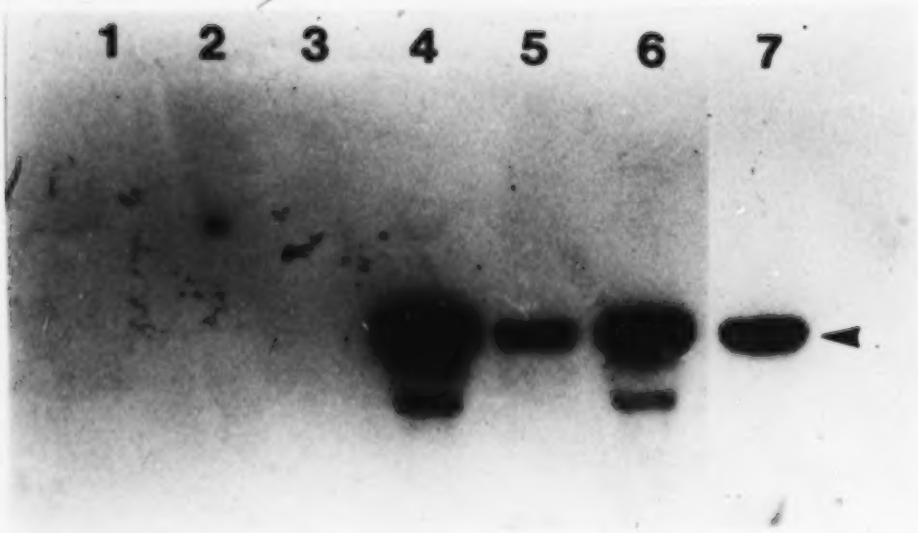

1
2
3
4
5
6
7

Fig. 12 Northern hybridization analysis of the tRNAs from the yeast transformant strains, assaying for the presence of Drosophila tRNA species after loss of suppressor activity. Transfer RNA was prepared from the yeast strains as described in Fig. 11, except that it was done after selecting for loss of the plasmid and suppression. The probe used was SPA#1 (Fig. 6).

Lane 1: *E. coli* tyrosine tRNA used as the size marker, in the ethidium bromide stained gels,

Lane 2: tRNA from yeast transformed with wild type $tRNA^{Ser}$ gene of *D. melanogaster* (before selection for loss of the plasmid),

Lane 3: tRNA from yeast transformed with just the vector, YRp7,

Lanes 4-7: Transfer RNA from yeast after selection for loss of the plasmid(s) carrying the genes for wild type (lane 4), opal (lane 5), ochre (lane 6) and amber (lane 7) suppressor tRNAs.

1 2 3 4 5 6 7

8. Subcloning into Carnegie20, an *E. coli-Drosophila* shuttle vector

For the purpose of introduction of the nonsense suppressor tRNA genes into the Drosophila genome, Carnegie20 (Rubin and Spradling, 1983) was used as a vehicle. It contains the β-lactamase gene for selection in *E. coli*, and the wild type sequence of rosy, (the structural gene for xanthine dehydrogenase; Xdh), for selection in Drosophila. Fig. 13 indicates the cloning strategy used to construct the recombinant derivatives pSS193, pSS06, pSS11f, and pSS35 (described in Materials and Methods).

A 2.1 kb *Sal*I-*Xho*I fragment containing the appropriate wild type or mutant tRNA gene was cloned into the *Sal*I site of the Carnegie20 vector. All recombinant plasmids were identified in *E. coli* by ampicillin resistance selection, followed by colony hybridization to appropriate end-labeled oligonucleotides (Fig. 6). The size of the fragment inserted into each of the selected clones (pSS193, pSS06, pSS11f, and pSS35) was confirmed by restriction endonuclease mapping and Southern hybridization (Fig. 14) to the 'a14' oligonucleotide (Fig. 6). As indicated in Fig. 14A, a 2.1 kb fragment corresponding to the fragment containing the tRNA gene (wild type or mutant derivative) was seen only in the case of the recombinant clones. The hybridization of this 2.1 kb fragment to the end-labeled 'a14' probe is demonstrated in Fig. 14B. The specificity of hybridization further proves the identity of the 2.1 kb fragments seen in the agarose gel. The sequence of each mutant gene in each of the clones was again confirmed by supercoiled plasmid sequencing (Chen and Seeburg, 1985).

9. Germline transformations of Drosophila

All the above recombinant derivatives of Carnegie20 were introduced into the Drosophila genome by microinjections of preblastoderm embryos (Rubin and Spradling, 1982; Spradling and Rubin, 1982), with one of the Carnegie20 constructs and a helper plasmid, pπ25.7wc. The latter was used as a source of the transposase for the Carnegie20 constructs. The advantage of using pπ25.7wc as the helper plasmid is that it cannot

Fig. 13 Schematic diagram representing the strategy used to clone the wild type and the nonsense suppressor derivatives of the $tRNA^{Ser}$ gene of *D. melanogaster* into the *E. coli*-Drosophila shuttle vector, Carnegie20. On the top is the 2.4 kb *Pst*I-*Hin*dIII *D.melanogaster* DNA fragment present in the M13 mp18 vector (containing either the wild type $tRNA^{Ser}_7$ gene or its mutant derivatives) from which, a 2.1 kb *Sal*I-*Xho*I fragment was cloned into the unique *Sal*I site in Carnegie 20, as indicated. The vector diagram (Carnegie 20) is from Pouwels et al. (1988). In the Carnegie 20 shuttle vector, the open areas represent the sequences derived from pUC8 plasmid, and the shaded portions represent the sequences as indicated in the figure. The letter, P in the shaded area represents the sequences from the P element .

Hind III
Xho I
Aval
Pvu II
Cla I
Sal I
Pst I
Hind III
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
10.8/0
amp
ori
Cosmid-30
6.6 kb

Fig.14A Agarose gel (1.0%) electrophoresis of restriction digests of Carnegie 20 recombinant clones containing the wild type $tRNA^{Ser}$ gene and its suppressor derivatives. The arrow indicates the 2.1 kb *Sal*I-*Xho*I fragment cloned into the Carnegie 20 vector. The migration of the fragments is from top to bottom and the various lanes contain DNA of the following identities:

Lane 1: Lambda DNA digested with *Hin*dIII (the sizes of the fragments, from top to bottom, are as follows: 23.13 kb, 9.416 kb, 6.557 kb, 4.361 kb, 2.322 kb, 2.027 kb);

Lane 2: Linearized M13mp18 phage vector;

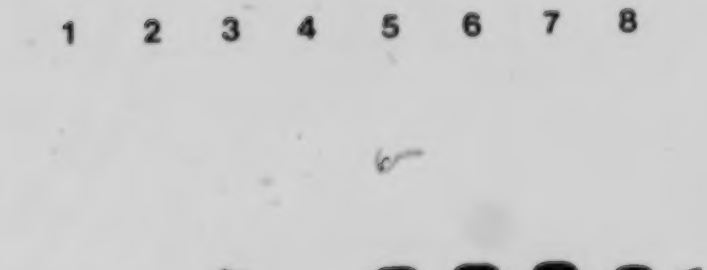
Lane 3: M-PH1 (described in Materials and Methods) digested with *Sal*I and *Xho*I;

Lane 4: Carnegie20 digested with *Sal*I and *Bam*H1;

Lanes 5-8: pSS193, pSS06, pSS11f, and pSS35 (all Carnegie20 derivatives) digested with *Sal*I and *Bam*H1;

Fig. 14B Autoradiograph of a Southern hybridization blot of the agarose gel in Fig.14A, probed with 5' end-labeled (with [γ-^{32}P]ATP, 4500Ci/m mol) 'a14' oligonucleotide probe that recognizes the 5' (-10 region, Fig.6) region of the $tRNA^{Ser}$ gene of *D. melanogaster*. The arrow denotes the same fragment as in 'A' above.

A



B

integrate into the Drosophila genome by itself, because it lacks one of the end repeat sequences required for integration.. The host strain used in the injection experiments was *b cn;ry*^{8} (described in Materials and Methods,). This strain was used as the recipient of injected DNA, instead of the appropriate nonsense mutation carrying strain, since it has been previously observed that the *b cn;ry*^{8} is easier to transform (C. Molnar, unpublished observations). All the injections were carried out as described in Materials and Methods section 21, and the results are tabulated in Table 4.

The embryos were dechorionated and desiccated prior to injections. Initially, chemical dechorionation was done (with Chlorox liquid bleach), but later the embryos were mechanically dechorionated under high humidity conditions. Mechanical dechorionation resulted in higher survival to hatching (30-50%) compared to chemical dechorionation (10% survival to hatching). Constructs pSS11f and pSS35 were injected entirely into mechanically dechorionated embryos. The volume of DNA injected was estimated to be approximately 25 picoliters. This estimate was based on the previous reports of Molnar (1985) and Spencer (1987). The injected embryos were kept at 18° C under halocarbon oil, and transferred to standard fly medium (Nash and Bell, 1968) immediately after hatching. G_0's and G_1's were independently crossed to flies of the parental genotype (Fig. 15) and from G_2 onwards, the ry^+ transformants were maintained by intercrossing.

Approximately 5-10% of the injected embryos reached the adult stage and 20-50% of these showed transient expression of the plasmid (ry^+ phenotype) (Table 4). Among all G_0 adults, 38-60% were found to be fertile, while 2-12.5% of these G_0 adults gave G_1 ry^+ flies. It is also quite evident from Table 4 that the lowest percent of G_1 transformed lines was seen in the case of the opal construct (pSS06) and the highest was seen for the case of the amber (pSS35) construct. Except for the pSS06 transformants, all others were found to be quite healthy. The details of the G_1 transformed lines are presented in Tables 5-8 respectively for the wild type (pSS193), opal (pSS06), ochre (pSS11f), and amber (pSS35) constructs.

Table 4: Microinjection series of the wild type and mutant $tRNA^{Ser}$ gene constructs into strain *b cn;ry*^{8} of *D. melanogaster*.

construct[a]	#embryos injected	#hatched	#3rd instars	#G_0 adults	#ry^+G_0	#G_0 adults fertile (both ry^+&ry^-)	#G_0 that gave $G_1 ry^+$	%ry^+ in G_1
pSS193	1235	257	41	26	14	15	4	1-16
pSS06	1548	287	57	40	20	23	1	6.6
pSS11f	875	213	55	34	13	19	1	55
pSS35	600	208	58	40	9	24	5	4-49.13

a. The constructs used in the injection experiments contain the wild type (pSS193) $tRNA^{Ser}_7$ gene or its opal (pSS06), ochre (pSS11f), or amber (pSS35) derivatives (Fig.13).

Fig. 15 Schematic representation of the crossing strategy followed to select and maintain the germline transformants of *D. melanogaster*.

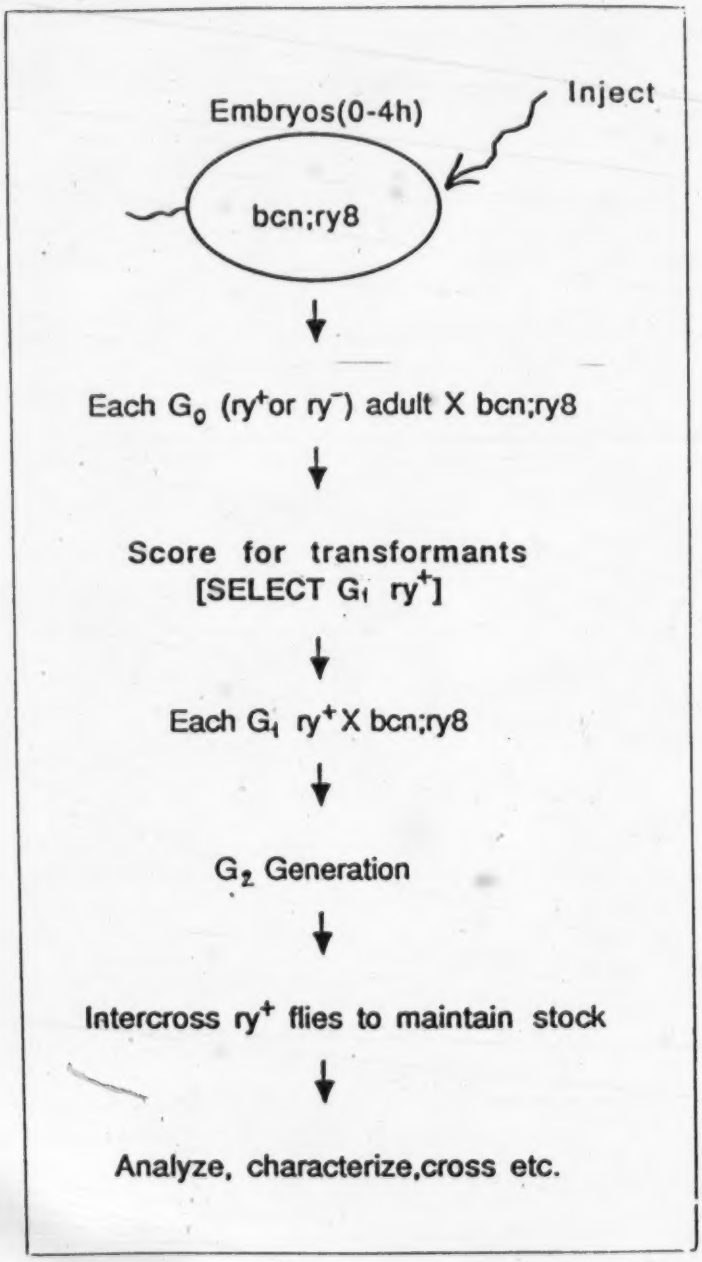

Embryos(0-4h)
Inject
bcn;ry8
Each G_0 (ry^+or ry^-) adult X bcn;ry8
Score for transformants
[SELECT G_1 ry^+]
Each G_1 ry^+ X bcn;ry8
G_2 Generation
Intercross ry^+ flies to maintain stock
Analyze, characterize,cross etc.

Table 5: Injection data for the plasmid pSS193 (containing the $tRNA_{UCN}^{Ser}$ gene)[a].

G_o adult	G_o sex	G_o ry^+?	G_o Fertility	#G_1	#G_1 ry^+	#G_1 ry^-
W1	F	no	F	10	0	10
W2	F	yes	F	101	14	87
W3	F	no	S	--	--	--
W4	M	yes	S	--	--	--
W5	M	yes	F	11	0	11
W6	F	no	F	52	7	45
W7	F	no	S	--	--	--
W8	M	yes	F	12	0	12
W10	M	yes	F	32	0	32
W11	F	no	S	--	--	--
W12	F	yes	S	--	--	--
W13	F	yes	F	30	0	30
W15	F	yes	F	146	0	146
W16	M	no	S	--	--	--
W17	M	yes	S	--	--	--
W18	M	no	F	116	0	116
W19	M	yes	S	--	--	--
W20	M	no	F	23	0	23
W22	M	yes	F	101	0	101
W24	M	no	F	149	3	146
W25	F	yes	F	110	1	109
W26	M	no	S	--	--	--
W27	F	no	F	117	0	117
W31	M	yes	S	--	--	--
W33	F	yes	S	--	--	--
W39	F	no	F	10	0	10

[a] The plasmids were injected into b cn; ry8 embryos at a concentration of 0.22 µg/µl along with 0.1µg/µl of helper plasmid.

Table6: Injection data for the plasmid pSS06 (containing the opal suppressor $tRNA^{Ser}$ gene)[a].

G_o adult	G_o sex	G_o ry^+?	G_o fertility	#G_1	#G_1 ry^+	#G_1 ry^-
S2	M	yes	F	35	0	35
S3	F	yes	F	83	0	83
S4	F	yes	S	--	--	--
S7	F	no	S	--	--	--
S8	F	no	F	12	0	12
S10	M	no	F	46	0	46
S11	M	no	F	76	0	76
S12	M	no	S	--	--	--
S13	F	no	F	14	0	14
S15	M	no	F	193	0	193
S16	F	no	F	101	0	101
S17	M	no	F	93	0	93
S18	F	yes	S	--	--	--
S19	F	no	F	97	0	97
S20	M	yes	F	13	0	13
S21	M	yes	S	--	--	--
S22	M	no	F	50	0	50
S24	M	yes	F	129	0	129
S25	F	yes	F	124	0	124
S26	F	no	F	7	0	7
S27	M	no	F	93	0	93
S33	M	yes	S	--	--	--
S34	M	yes	S	--	--	--
S36	M	yes	F	61	4	57
S37	M	yes	S	--	--	--
S38	F	no	F	95	0	95
S39	M	no	S	--	--	--
S40	M	no	S	--	--	--
S41	M	no	F	10	0	10
S42	F	yes	S	--	--	--
S43	F	yes	S	--	--	--
S45	M	no	F	20	0	20
S46	F	yes	F	78	0	78
S48	F	yes	S	--	--	--
S51	F	yes	F	155	0	158
S52	M	no	S	--	--	--
S53	F	yes	F	59	0	59
S55	F	no	S	--	--	--
S56	F	yes	S	--	--	--
S57	F	no	S	--	--	--

The plasmids were injected into b cn; ry8 embryos at a concentration of 0.22 µg/µl along with 0.1µg/µl of helper plasmid.

Table7: Injection data for the plasmid pSS11f (containing the ochre suppressor $tRNA^{Ser}$ gene).

G_0 adult	G_0 sex	G_0 ry^+?	G_0 fertility	#G_1	#G_1 ry^+	#G_1 ry^-
C1	F	no	F	103	0	103
C2	M	no	F	323	0	323
C3	F	no	S	--	--	--
C4	F	no	S	--	--	--
C5	F	no	F	82	0	82
C6	M	no	F	92	0	92
C7	M	no	F	179	0	179
C8	M	no	F	195	0	195
C9	M	no	F	163	0	163
C10	F	no	S	--	--	--
C11	F	yes	S	--	--	--
C12	F	yes	F	201	0	201
C13	F	yes	S	--	--	--
C14	F	no	S	--	--	--
C15	M	no	S	--	--	--
C16	F	no	F	124	0	124
C17	F	yes	S	--	--	--
C18	M	no	F	150	0	150
C19	M	no	F	200	0	200
C20	M	yes	F	155	0	155
C21	F	yes	F	31	0	31
C22	F	yes	S	--	--	--
C23	F	no	F	130	0	130
C24	F	no	S	--	--	--
C25	M	no	F	120	0	120
C26	M	no	S	--	--	--
C27	M	yes	S	--	--	--
C28	M	yes	F	84	26	58
C29	M	yes	S	--	--	--
C30	M	no	F	313	0	312
C31	M	no	F	280	0	280
C32	F	yes	S	--	--	--
C33	M	yes	F	53	0	53
C34	M	yes	S	--	--	--

The plasmids were injected into b cn; ry8 embryos at a concentration of 0.22 µg/µl along with 0.1µg/µl of helper plasmid.

Table 8: Injection data for the plasmid pSS35 (containing the amber suppressor $tRNA^{Ser}$ gene).

G_o adult	G_o sex	G_o ry^+?	G_o fertility	#G_1	#G_1 ry^+	#G_1 ry^-
M1	M	no	S	--	--	--
M2	F	yes	S	--	--	--
M3	F	no	S	--	--	--
M4	M	yes	S	--	--	--
M5	M	no	F	176	0	176
M6	M	no	F	6	0	6
M7	F	yes	F	2	0	2
M8	F	yes	S	--	--	--
M9	F	no	F	161	0	161
M10	F	no	S	--	--	--
M11	F	no	S	--	--	--
M12	F	no	F	39	0	39
M13	F	no	S	--	--	--
M14	M	yes	F	123	0	123
M15	M	yes	F	369	128	241
M16	F	yes	F	41	9	32
M17	M	no	S	--	--	--
M18	M	no	S	--	--	--
M19	M	no	F	266	0	266
M20	M	no	S	--	--	--
M21	F	no	F	39	0	39
M22	F	no	S	--	--	--
M23	F	no	F	152	0	152
M24	F	no	S	--	--	--
M25	F	no	F	42	0	42
M26	F	no	F	25	1	24
M27	F	no	S	--	--	--
M28	F	no	S	--	--	--
M29	M	yes	F	281	0	281
M30	M	yes	F	80	0	80
M31	M	no	F	387	0	387
M32	M	no	S	--	--	--
M33	M	no	F	98	0	98
M34	M	no	F	207	0	207
M35	M	no	F	268	21	247
M36	F	no	F	77	0	77
M37	F	no	F	115	0	115
M38	M	no	F	235	0	235
M39	M	no	F	173	85	88
M40	F	no	F	20	0	20

The plasmids were injected into b cn; ry8 embryos at a concentration of 0.22 µg/µl along with 0.1µg/µl of helper plasmid.

The percentage of ry^+ flies among the G_1 flies ranged from 1-55% (Tables 5-8). The ry^+ phenotype shown by some of the G_0 flies is due to the transient expression of the plasmid constructs injected (Rubin & Spradling.,1982; Spradling & Rubin.,1982). The G_0 expression is consistent with previous reports (Chovnick et al., 1977; Girton et al., 1979), which indicated that a 1% level of expression of XDH was sufficient to restore normal eye color to flies. Only in the G_1 flies, does the ry^+ phenotype indicate the expression of a transposon integrated into the genome. To keep all of the integration events independent of each other, all the G_1 lines selected were maintained separately. Only one G_0 line gave G_1 ry^+ transformants for the opal construct (pSS06). Of the four G_1 ry^+ flies for that construct, only one (op1) could be maintained, since problems of sterility were encountered with the other three lines. No such problems were faced with respect to the other three sets of injections done. This is in contrast to the findings of other workers (Doerig et al., 1988; D. Garza and D. Hartl., 1987, personal communication) where high sterility levels were reported in the transformed flies. In these reports, the flies could not be maintained beyond the G_1 generation. In their work, no sterility problems were encountered when the wild type gene was injected, in agreement with the results reported here.

10. Genetic crosses of the germline transformant lines into an *Adh*⁻ background, and ADH assays

Both the opal and amber tester nonsense alleles in our collection are mutations in the structural gene for alcohol dehydrogenase. Since the recipient strain (*b cn;ry*$^{\delta}$) in all the transformation experiments has the wild type *Adh*$^+$ allele on its second chromosomes, the potential biological activity of the suppressor tRNAs introduced into the Drosophila genome could not be assayed. Thus, it was necessary to introduce these suppressor tRNA-carrying transposons into the appropriate Adh negative background by genetic crosses. Two crossing schemes were followed for this purpose, for both opal and amber

transformant lines, and these are schematically represented in Figs. 16 and 17. Since we do not presently have an ochre tester allele in our collection, it was not possible to cross the transformant with the ochre suppressor tRNA gene with any relevant strain.

Flies with the genotype *CyO^{nB} pr cn/b Adh n^{4}; ry/ry [ry+]* (hereafter referred to as Cy WT(II)) from Fig. 16, with both the nonsense mutation and suppressor tRNA, were assayed for ADH activity. Flies with the genotype *CyO^{nB} pr cn/ b Adh n^{4}; ry/ry* (hereafter referred to as Cy ry(II)) served as a negative control. If the Cy WT (II) flies showed any ADH activity, it would be indicative of the suppression of the nonsense mutation by the suppressor tRNA gene product. In such a case, the Cy ry(II) flies should not show any ADH activity, due to the absence of the suppressor tRNA gene.

The first crossing scheme would reveal ADH activity due to the presence of active suppressor tRNA, but it would not indicate if the activity was due to the suppression of the opal (*Adh^{nB}*) mutation or the suppression of the amber (*n^{4}*) nonsense mutation. In order to be able to ascertain whether codon specificity was retained, the second cross (Fig. 17) was also designed. In this cross, flies with the phenotype Cy WT(I) should show activity with either an amber or an opal suppressor tRNA gene, while the flies with the phenotype Cy^{+} WT(I) should only be ADH positive if suppression of the amber allele occurs. However, these crossing schemes would not allow the integration events on the second chromosome to be assayed for suppression. This is due to the fact that in such cases the transposon carrying chromosome also carries the wild type allele for the *Adh* locus and therefore would be eliminated or absent in the genotypes selected.

All the selected transformed lines, were assayed for ADH activity as in Grell et al. (1968) after crossing the suppressor containing transposon into the appropriate Adh negative background, as described Figs. 16 and 17. One to five flies of the appropriate genotype were squashed onto Whatman 3 MM paper squares as described in Materials and Methods (section 22). 2-butanol was used as the substrate for the ADH enzyme in these assays. In this reaction, electrons are transferred from the alcohol through NAD^{+} and

Fig. 16 Schematic representation of the crossing strategy followed to introduce the suppressor tRNA genes into the ADH negative genetic background with the appropriate Adh nonsense mutation. The designation II (for eg., Cy ry II) in the figure represents the number assigned to the cross for practical purposes, and '(ry^+)' in the figure indicates the presence of the transposon, containing the $tRNA^{Ser}$ gene (wild type or mutant form of the gene) and the structural gene for the XDH enzyme, within the Drosophila genome. However, it does not indicate the genomic location of the transposon within the Drosophila genome.

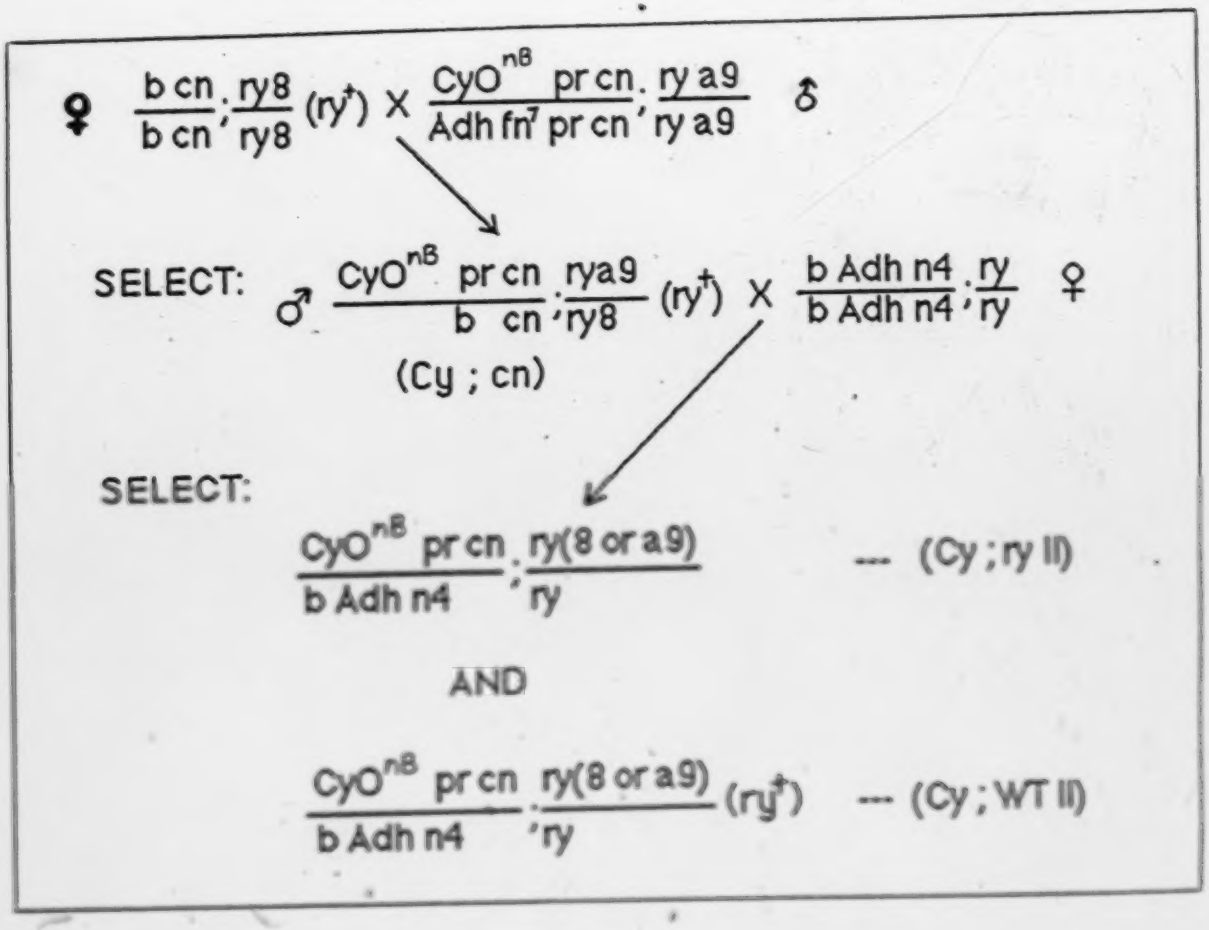
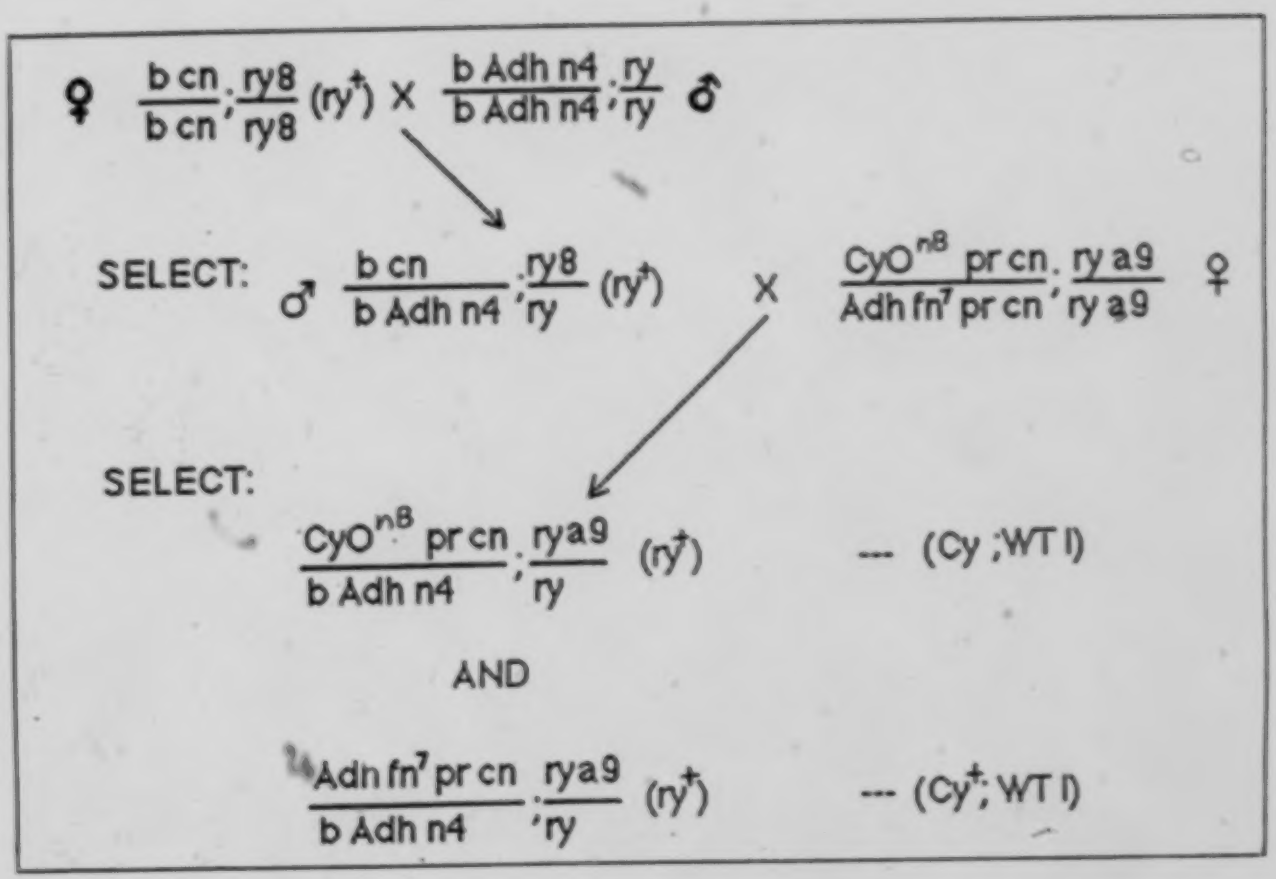

♀ b cn / b cn ; ry8 / ry8 (ry+) X CyO n8 pr cn / Adh fn7 pr cn ; ry a9 / ry a9 ♂
SELECT: ♂ CyO n8 pr cn / b cn ; rya9 / ry8 (ry+) X b Adh n4 / b Adh n4 ; ry / ry ♀
(Cy ; cn)
SELECT:
CyO n8 pr cn / b Adh n4 ; ry(8 or a9) / ry --- (Cy ; ry II)
AND
CyO n8 pr cn / b Adh n4 ; ry(8 or a9) / ry (ry+) --- (Cy ; WT II)

Fig. 17 Schematic representation of the alternative crossing strategy followed to introduce the suppressor tRNA genes into the ADH negative genetic background with the appropriate Adh nonsense mutation (Details discussed in text). The designation (for eg., Cy WT I) in the figure represents the number assigned to the cross for practical purposes, and '(ry^+)' in the figure indicates the presence of the transposon, containing the $tRNA^{Ser}$ gene (wild type or mutant form of the gene) and the structural gene for the XDH enzyme, within the Drosophila genome. However, it does not indicate the genomic location of the transposon within the Drosophila genome.


♀ b cn / b cn ; ry8 / ry8 (ry+) X b Adh n4 / b Adh n4 ; ry / ry ♂
SELECT: ♂ b cn / b Adh n4 ; ry8 / ry (ry+) X CyO n8 pr cn / Adh fn7 pr cn ; ry a9 / ry a9 ♀
SELECT:
CyO n8 pr cn / b Adh n4 ; ry a9 / ry (ry+) --- (Cy ; WT l)
AND
Adh fn7 pr cn / b Adh n4 ; ry a9 / ry (ry+) --- (Cy+ ; WT l)

phenazine methosulfate (PMS) to nitroblue tetrazolium (NBT) reducing it to an indigo formazan dye. Therefore, in these assays appearance of a dark purple color indicates ADH activity. When the transformed lines were assayed for *in vivo* suppression of the Adh nonsense mutation by the *in vitro* constructed opal and amber suppressor tRNAs, no detectable ADH activity was found. The assays were repeated using a spectrophotometric assay (Spencer, 1987) where reduction of NAD^+ was monitored at 340 nm in the presence of 2- butanol as the substrate in crude fly extracts (prepared as described in Materials and Methods). This assay did not detect ADH activity in any of the lines tested. Assays were performed with diluted wild type extracts. They indicated that approximately 1% of wild type activity could have been detected in the suppressor containing lines.

11. Genomic Southern analysis of the transformed lines

To verify that the ry^+ phenotype of the transformed lines was not due to a reversion of the original mutation and to prove that the transposon carrying the suppressor tRNA was actually integrated into the Drosophila genome, Southern hybridization analyses of genomic DNA from the selected transformants were done. Genomic DNAs were prepared as described in Materials and Methods (section 23). Five micrograms of genomic DNA, digested with appropriate restriction endonuclease(s), were run in each lane and capillary blotted onto GeneScreen Plus membranes. The restriction endonucleases chosen were such that the recognition site for one of them (*Sal*I) was on the endogenous DNA fragment that was cloned into the Carnegie20 vector (Fig.13). The recognition site for the other enzyme (*Bam*HI) was present just external to the fragment cloned into Carnegie20, within the cloning cassette of the vector (Fig. 13). There was no *Bam*HI recognition site in the cloned genomic DNA fragment carrying the tRNA genes. Therefore, the double-digestion would ensure that exactly the same sized fragment as was cloned into Carnegie20 would be seen in the transformed lines, but not in the untransformed control lanes. This is because the closest *Bam*HI recognition site within the genomic DNA corresponding to the cloned

fragment would be at a random location, and therefore would be seen as a different sized fragment. Also, this fragment should be seen in both transformed and untransformed lines. The Gene-Screen Plus membranes were probed with the 2.1 kb *Sal*I-*Xho*I fragment (Fig. 5) which was labeled with [α–^{32}P]dCTP.

The expected 2.1 kb DNA fragment was detected in the lanes containing DNA from transformed lines (Fig. 18). This proves that all the lines exhibiting the ry^+ phenotypes were, indeed, germline transformants. In addition to the 2.1 kb fragment, another band of the same intensity and approximately 5 kb in size was seen in all the lanes, including the untransformed control. This band corresponds to the fragment containing the endogenous copy of this specific $tRNA^{Ser}$ gene. Since it has the same flanking sequences, the homology of this fragment with the probe used was high, and so was the intensity of the signal. The other fainter bands correspond to the other $tRNA^{Ser}$ genes from other parts of the genome. The different intensities of the bands between the lanes could be due to minor differences in the amounts of DNA loaded into each lane. However, in general, the intensity of these bands is less because of the low homology between the flanking sequence of the probe with the regions of the Drosophila genome containing the other $tRNA^{Ser}$ genes.

12. Copy number of transposons in the transformed lines

The Southern hybridization analysis performed in section 11 would only indicate whether the 2.1 kb fragment was present in the Drosophila genome. However, it will not provide information regarding the number of copies that are present in each genome. In order to estimate the copy number, another genomic Southern hybridization analysis was done using a different strategy. The various genomic DNAs were digested with *Xho*I restriction endonuclease. There is only one *Xho*I recognition site in the entire transposon (see Fig. 13) in each transformed line. If there is more than one copy of the transposon in any of the transformed lines, the sites of their integration would be different. In such a

Fig. 18 Genomic Southern hybridization analysis of the transformed Drosophila lines. 5.0 μg of genomic DNA, digested with *Sal*I and *Bam*H1, were loaded into each lane. The digests were electrophoresed on a 1.0% agarose gel at 36 volts for 16 hours with buffer circulation. The DNA was capillary blotted onto a GeneScreen Plus nylon membrane and probed with the 2.1 kb *Sal*1-*Xho*1 fragment (Fig. 5) containing the $\overline{\text{tRNA}^{\text{Ser}}}$ gene of *D. melanogaster*.

Lane 1: Genomic DNA digest of 'wt4';

Lane 2: Genomic DNA digest of 'oc3';

Lane 3: Genomic DNA digest of 'am5';

Lane 4: Genomic DNA digest of 'am8';

Lane 5: Genomic DNA digest of 'am13';

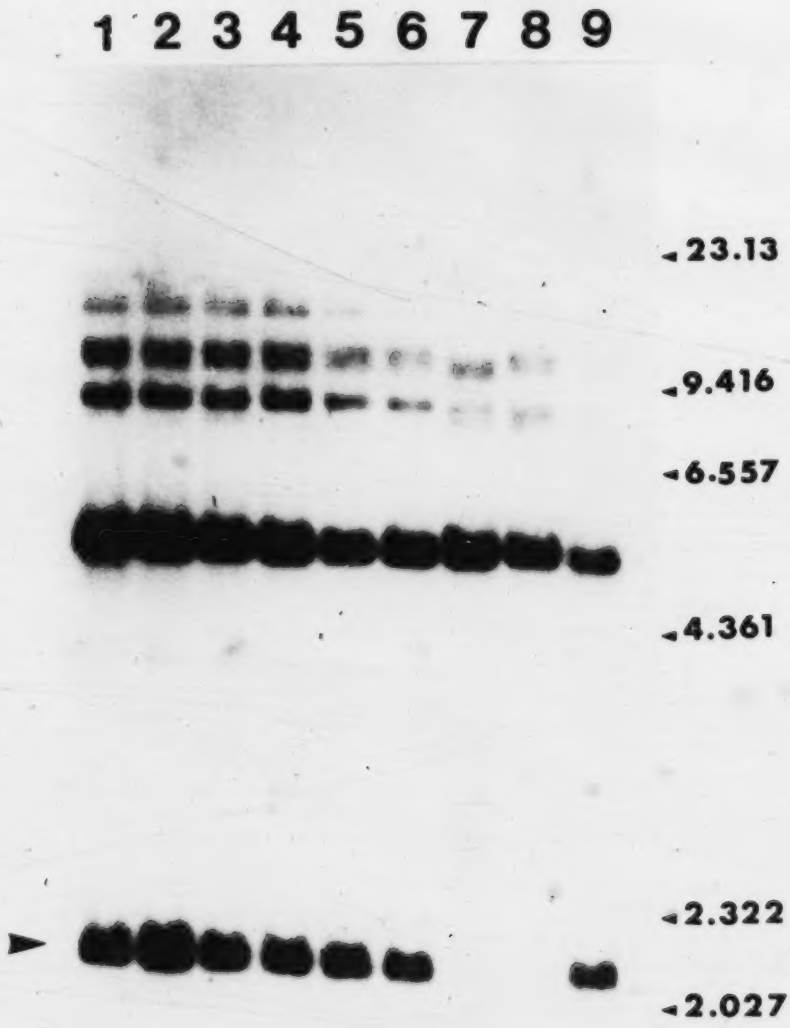
Lane 6: Genomic DNA digest of 'op1';

Lane 7: Genomic DNA digest of untransformed control;

Lane 8: Genomic DNA digest of untransformed control;

Lane 9: Genomic DNA digest of 'op1' using approximately half the amount of DNA as in the other lanes.

The arrow indicates the expected 2.1 kb band.


1 2 3 4 5 6 7 8 9
23.13
9.416
6.557
4.361
2.322
2.027

situation, for each site of integration there is a high probability that the closest endogenous *Xho*I site in each case will be a different distance from the *Xho*I site in the integrated transposon, thus producing one extra band per integration event. When probed with the 2.1 kb fragment that was cloned into Carnegie20, it was found that there was only one copy of the transposon per transformed line (Fig. 19). The common bands corresponding to the endogenous $tRNA^{Ser}$ genes are indicated by arrowheads. In the 'am5' and 'am13' lanes, one of the two common bands was found missing. To check if this was due to polymorphism for *Xho*I sites, two more independent and separate preparations (Fig. 19, lanes 8 and 9) of the control (untransformed) DNA were made and were run in the same gel. As can be seen from Fig. 19, the common band pattern of one of them (lane 8) correlates with those of 'am5' and 'am13', lending support to the idea of *Xho*I site polymorphism in Drosophila genomic DNAs. Other than the common bands, only one extra band was seen in each of the lanes containing DNA from transformed lines indicating that there was only one copy of the transposon per line.

13. Localization of the transposon on the Drosophila chromosomes

The Southern hybridization analyses, done to determine the copy number of the transposon constructs, indicated one copy per genome for each transformed Drosophila line. In order to confirm and characterize this further, *in situ* hybridizations to salivary gland chromosomes were done. Moreover, genetic crosses done as described in section 10 of this Chapter allowed the identification of the chromosome into which the integration event occurred. The data from both the genetic crosses and *in situ* analysis led to the conclusions tabulated in Table 9.

Line 'wt4' has the transposon on the third chromosome; lines 'op1', 'oc3', and 'am13' have it on the X chromosome; and 'am5' and 'am8' were found to have the integration into the second chromosome of the Drosophila genome. The strategy followed to identify their location using genetic crosses (cross I, Fig. 16) was as follows: The first

Fig. 19 Genomic Southern hybridization analysis of the Drosophila transformed lines to determine the copy number of transposons in each line.

Five micrograms of genomic DNA digested with *Xho*I were loaded into each lane. The digests were electrophoresed on a 1.0% agarose gel at 36 volts for 16 hours with buffer circulation. The DNA was capillary blotted onto a GeneScreen Plus nylon membrane and probed with the 2.1 kb *Sal*I-*Xho*I fragment (Fig. 5)

Lane 1: Genomic DNA digest of untransformed control;

Lane 2: Genomic DNA digest of 'wt4';

Lane 3: Genomic DNA digest of 'oc3';

Lane 4: Genomic DNA digest of 'am5';

Lane 5: Genomic DNA digest of 'am8';

Lane 6: Genomic DNA digest of 'am13';

Lane 7: Genomic DNA digest of 'op1';

Lane 8: Genomic DNA digest of untransformed control preparation#2

Lane 9: Genomic DNA digest of untransformed control preparation#3

The arrowheads indicate the common bands between the transformed and untransformed lines, taking into account the polymorphisms discussed in the text. The very faint bands in most lanes probably represent a slight level of cross hybridization with endogenous copies of the other $tRNA^{Ser}$ genes having different flanking sequences.

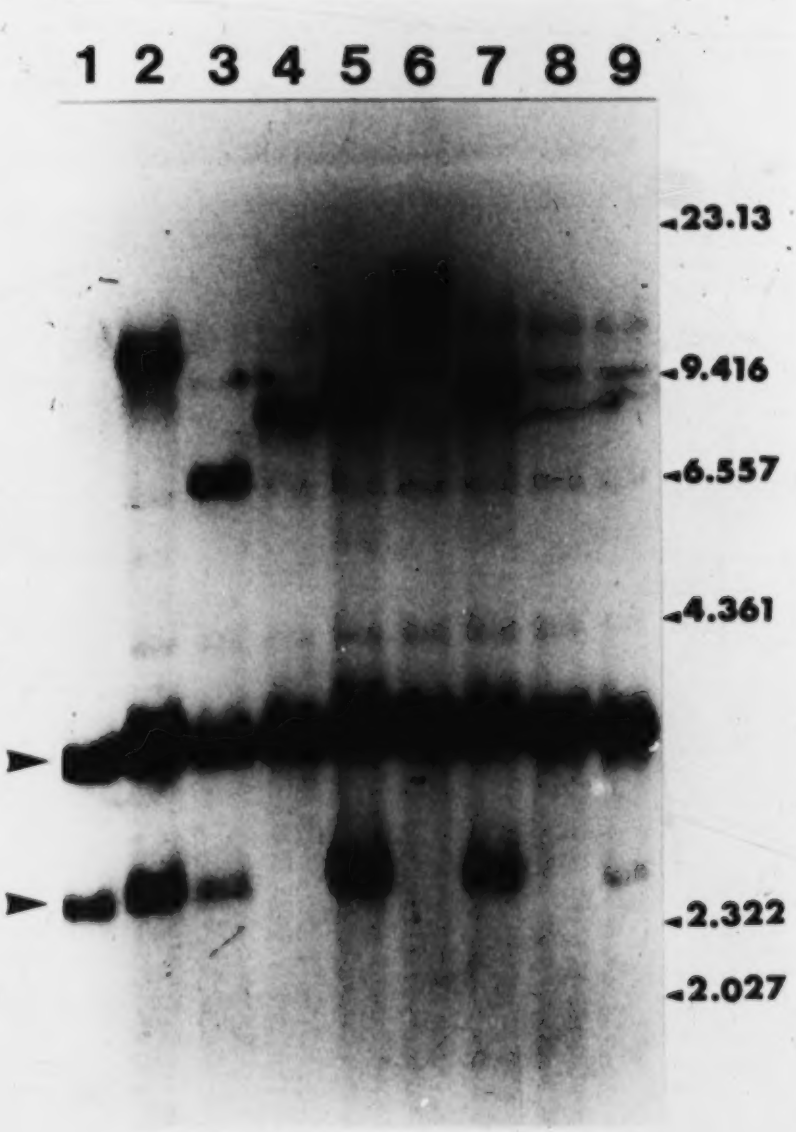

1 2 3 4 5 6 7 8 9
23.13
9.416
6.557
4.361
2.322
2.027

Table9: Summary of the locations of the transposons within the *Drosophila* genome.

		Location determined	
Line#	Transformed line[a]	by genetic crosses	by in situs
1	'wt4'	III	---
2	'op1'	X	X [19D]
3	'oc3'	X	X [10B]
4	'am5'	II	II [60E]
5	'am8'	II	II [30E]
6	'am13'	X	---

a. The transformed lines contain one extra wild type $tRNA^{Ser}$ gene ('wt4'), or its opal ('op1'), ochre ('oc3'), or amber ('am5', 'am8', 'am13') derivatives.

generation cross revealed any integration events on the X-chromosome by the typical 'criss-cross' inheritance pattern exemplified by any X-linked character. The II and III chromosome events could be distinguished in the second generation cross. If the transposon (or ry^+ phenotype) segregated away from the 'Cy' phenotype, then it was on chromosome II , otherwise, it would be on chromosome III. In this way, it was ascertained which major chromosome had an integrated transposon in each of the lines. This method would have missed any integrations on chromosome IV, as they would have been grouped with the chromosome III class. The assignments made by genetic analyses were confirmed for some of the lines (as given in Table 9), by *in situ* hybridizations to salivary gland chromosomes (Pardue and Gall, 1975; Glew et al., 1986), which further narrowed down the location of the transposon on specific Drosophila chromosomes.

When *in situ* hybridizations to the salivary gland chromosomal preparations were done, one gland of the pair was probed with the nick-translated plasmid (pDt5), which recognizes the $tRNA^{Ser}$ gene sequences, and the other gland with the nick-translated Carnegie20 vector which recognizes the rosy gene sequences. Since the transposon contains both the $tRNA^{Ser}$ gene and the rosy gene, both probes should recognize one common region, and that was found to be the case. In addition, each probe recognized the other expected regions. The plasmid (pDt5) probe recognized the endogenous $tRNA^{Ser}$ gene sequences (band 23E on the left arm of the II chromosome), while Carnegie20 recognized the endogenous rosy sequences (band 87 on the right arm of the III chromosome). Carnegie20 also recognized white gene sequences (close to the tip of the X chromosome) in some cases. This is explainable since Carnegie20 contains a short segment of white gene sequences within it (Rubin and Spradling.,1983). Hybridization to the white sequences was not seen in all cases. This may be due to the period of hybridization. Since Carnegie20 has only a few hundred bases of the white gene sequence, the endogenous white gene sequences would not light up if the period of hybridization was too short.

In this manner, the transposon in line 'op1' was localized to band 19D on the X chromosome (Fig. 20) which is close to the chromocentre. In line 'oc3' (Fig. 22), it was also localized on the X chromosome but at band 10B. In lines 'am5' (Fig. 21) and 'am8', the transposon was found to be on the II chromosome. In the former, it was on the right arm of II chromosome at band position 60E, almost at the tip of the right arm. In the latter, it was at band 30E on the left arm of the II chromosome. These results are consistent with those found by the genetic crosses.

14. Genomic Southern analyses with oligonucleotide probes

The data in sections 11, 12, and 13 revealed the presence of the 2.1 kb DNA fragment in the Drosophila genomes of putative germline transformants. However, those analyses fail to determine if the tRNA gene sequence was fully retained within that 2.1 kb fragment. There are precedents for integrated P-element transposons with various internal deletions (reviewed in Williams et al., 1988). Therefore, to confirm that the tRNA gene was not deleted, another genomic Southern analysis was done. The restriction endonucleases used, as well as the method of electrophoresis and blotting were exactly the same as in section 11, except that 10 μg of genomic DNA were loaded into each lane and a 5' end-labeled oligonucleotide (SPA#1, Fig. 6) was used as a probe instead of the 2.1 kb fragment. The oligonucleotide SPA#1 recognizes the region of the $tRNA^{Ser}$ that is 3' to the anticodon, and represents a much more specific probe for the tRNA gene as compared to the entire 2.1 kb fragment. The hybridization and wash conditions are described in section 25 of Materials and Methods. The Southern analysis (Fig. 23) revealed exactly the same pattern as was previously shown in Fig. 18, confirming that the 2.1 kb fragment introduced into the Drosophila genome retained the much smaller (82 bp) tRNA gene. The major difference in Fig. 23 compared to Fig. 18 is that all the bands are now of approximately equal intensity. Since the probe used was an oligomer complementary to common sequences in all serine tRNA genes, it is expected to recognize all with equal

Fig. 20. *In situ* hybridization analyses of 'op1'. Salivary gland squashes, *in situ* hybridization, autoradiography and photography were performed as described in Materials and Methods.

In Fig.20A, the preparation from one gland of the pair of salivary glands was probed with nick translated Carnegie20 which contains the ry^+ gene cloned into the Carnegie2 vector. This vector also contains Drosophila white gene sequences (details given in Fig.13). In the figure, 'W', 'ry', and 'tr' indicate the areas of hybridization to the endogenous white locus on the X chromosome, the endogenous rosy locus on the right arm of the III chromosome, and the rosy sequences on the transposon on the X chromosome (band 19D), respectively.

In Fig.20B, the preparation from the other gland of the pair was probed with nick translated pDt5, which contains the $tRNA^{Ser}$ gene from band 23E of Drosophila II chromosome cloned into the pBR322 vector. As expected, two areas of hybridization, one to the endogenous $tRNA^{Ser}$ gene on the II chromosome (23E), and the other to the copy of the same gene carried on the transposon (tr), were seen (indicated by the arrowheads).

A
B

Fig. 21. *In situ* hybridization analyses of 'am5'. Salivary gland squashes, *in situ* hybridization, autoradiography and photography were performed as described in Materials and Methods.

In Fig.21A, the preparation from one gland of the pair of salivary glands was probed with nick translated Carnegie20 which contains the ry^+ gene cloned into the Carnegie2 vector. This vector also contains Drosophila white gene sequences (details given in Fig.13). In the figure, 'W', 'ry', and 'tr' indicate the areas of hybridization to the endogenous white locus on the X chromosome, the endogenous rosy locus on the right arm of the III chromosome, and the rosy sequences on the transposon on the II chromosome (band 60E), respectively.

In Fig.21B, the preparation from the other gland of the pair was probed with nick translated pDt5, which contains the $tRNA^{Ser}$ gene from band 23E of Drosophila II chromosome cloned into the pBR322 vector. As expected, two areas of hybridization, one to the endogenous $tRNA^{Ser}$ gene on the II chromosome (23E), and the other to the copy of the same gene carried on the transposon (tr), were seen (indicated by the arrowheads).

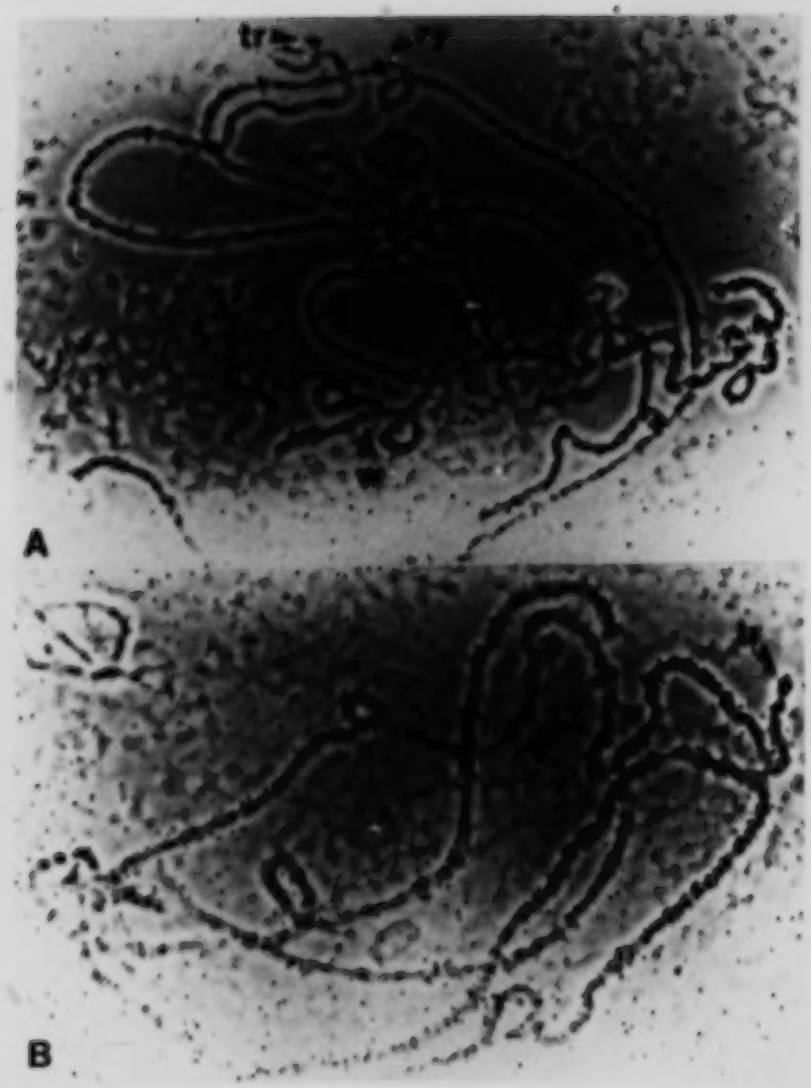

A
B

Fig. 22. *In situ* hybridization analyses of 'oc3'. Salivary gland squashes, *in situ* hybridization, autoradiography and photography were performed as described in Materials and Methods.

In Fig.22A, the preparation from one gland of the pair of salivary glands was probed with nick translated Carnegie20, which contains the ry^+ gene cloned into the Carnegie2 vector. This vector also contains Drosophila white gene sequences (details given in Fig.13). In the figure, 'ry', and 'tr' indicate the areas of hybridization to the endogenous rosy locus on the right arm of the III chromosome, and the rosy sequences on the transposon on the II chromosome (band 30E), respectively. Hybridization to the white gene sequences was not seen in this case. This is probably due to insufficient period of hybridization as explained in the text.

In Fig.22B, the preparation from the other gland of the pair was probed with nick translated pDt5, which contains the $tRNA^{Ser}$ gene from band 23E of Drosophila II chromosome cloned into the pBR322 vector. As expected, two areas of hybridization, one to the endogenous $tRNA^{Ser}$ gene on the II chromosome (23E), and the other to the copy of the same gene carried on the transposon (tr), were seen (indicated by the arrowheads).

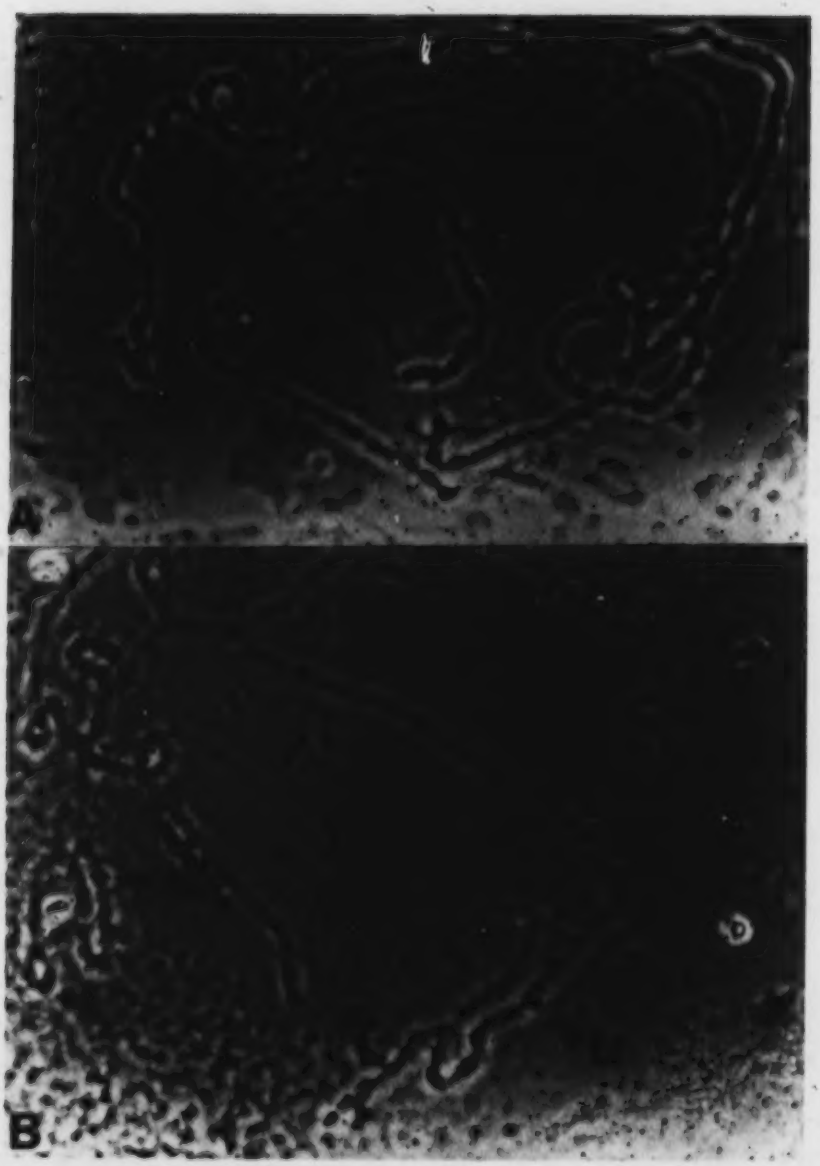

A
B

Fig. 23 Genomic Southern analysis of the transformed Drosophila lines, to determine the presence of the suppressor tRNA gene in the integrated transposon.

Ten micrograms of genomic DNA digested with *Sal*1 and *Bam*H1 were loaded into each lane. The digests were electrophoresed on a 1.0% agarose gel at 36 volts for 16 hours, with buffer circulation. The DNA was capillary blotted onto a GeneScreen Plus nylon membrane and probed with the 5' end-labeled SPA#1 (Fig. 6) probe.

Lanes 1-3: Genomic DNA digests of untransformed control strains;

lane 4: Genomic DNA digests of 'wt4';

lane 5: Genomic DNA digests of 'oc3';

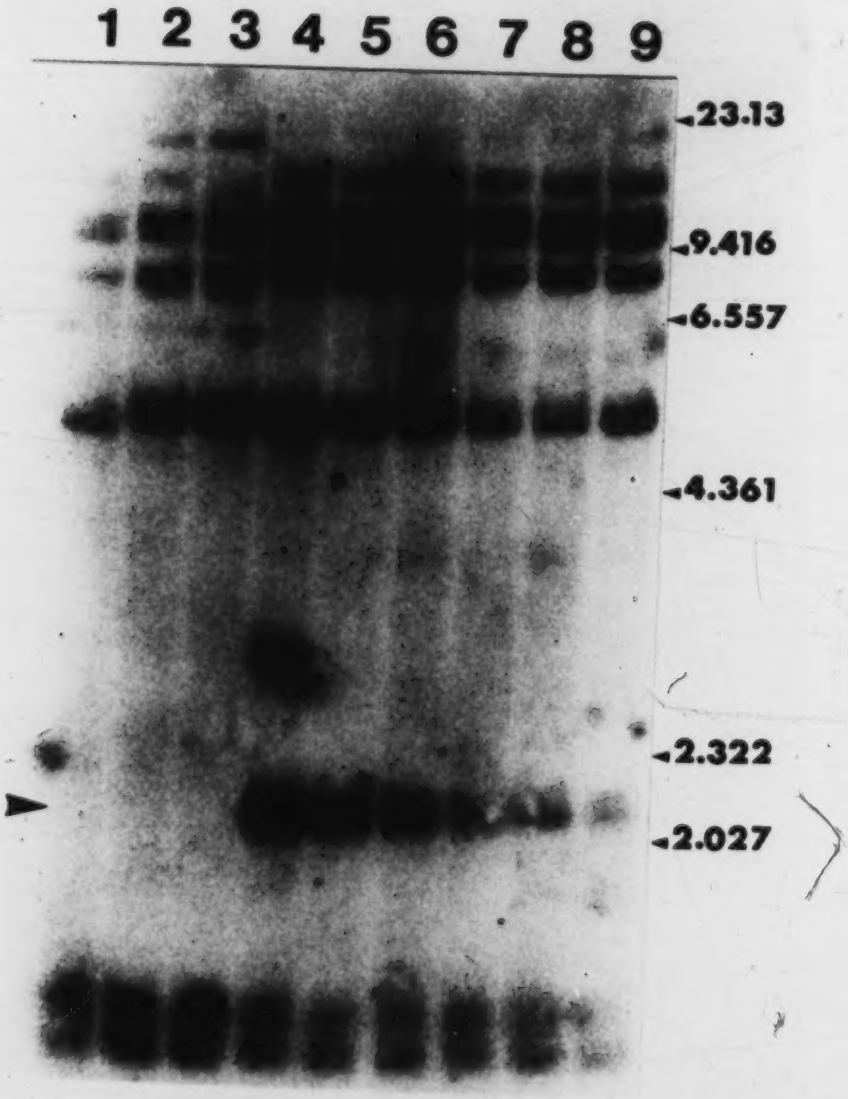
lane 6: Genomic DNA digests of 'am5';

lane 7: Genomic DNA digests of 'am8';

lane 8: Genomic DNA digests of 'am13';

lane 9: Genomic DNA digests of 'op1';

The arrowhead indicates the band containing the tRNA gene from the transposon.


1 2 3 4 5 6 7 8 9
23.13
9.416
6.557
4.361
2.322
2.027

efficiency. The 2.1 kb band in lanes 7,8, and especially lane 9 were fainter than the other bands. Incomplete capillary transfer of DNA (probably due to air bubbles during blotting) could account for this difference. This is supported by the fact that these lanes contain DNA from the same preparation as lanes 5,6, and 7 respectively, in Fig. 18. Furthermore, when the same blot (as in Fig. 23) was stripped and re-probed with the 2.1 kb *Sal*I-*Bam*HI fragment, the same differences were seen. The other inconsistency is a 2.6 kb band in lane 4 of Fig. 23. Hybridization with the 0.6 kb *Eco*RI-*Hin*dIII probe (Fig. 13) revealed that to be due to incomplete digestions by the *Bam*HI restriction endonuclease. Repeated attempts to achieve complete digestion were unsuccessful.

15. Competition assays

Antibody competition assays were done to identify or determine if there was any increase in immunologically cross reacting material (CRM) in Adh negative mutants containing a suppressor tRNA gene in their genomes. The rationale is as follows. When the suppressor tRNA was introduced into the genome of Drosophila strains carrying the appropriate nonsense Adh allele, and assayed for ADH activity, no ADH activity was detected. If the suppressor tRNA was active and functional, serine would have been incorporated into the protein in response to the nonsense codon, leading to a full length protein. However, this may not result in ADH activity, in spite of the full length protein, if serine is not an appropriate amino acid at that position. Even though there was no ADH activity, a full length protein would lead to increased CRM levels in strains carrying the suppressor tRNA. Since these strains do not have any measurable ADH activity, a direct assay of CRM levels is not easy. Therefore, to circumvent this problem CRM levels were measured by a competition assay. Crude extracts from these strains (with and without suppressor tRNA gene) were mixed with wild type extracts and immunoprecipitations were done in the presence of anti-ADH polyclonal antibodies (generously provided by Dr. W. Sofer). The pellets were discarded and the activity remaining in the supernatant was

measured as described in Materials and Methods. In such a situation, strains carrying an active suppressor tRNA gene will produce CRM, albeit a biologically inactive protein, and thus titrate out more antibodies than those without the suppressor tRNA gene. Increased levels of CRM^+ product in a suppressor tRNA-containing strain would result in less wild type ADH activity being precipitated by the antibodies. Thus, if the suppressor containing strains do produce ADH CRM more wild type activity will remain in the supernatant of the mixed extracts after immunoprecipitation than when the wild type extract alone was used in the reaction. Under the conditions used a difference of one unit of ADH activity/min. can be detected after immunoprecipitation.

The titer of the antiserum was assayed by doing a titration curve (Fig. 24). Strain *b cn;ry*^{8} was used as the wild type control (with respect to the *Adh*$^+$ locus). To a constant volume of *b cn;ry*^{8} extract (100 units of ADH activity, defined in Materials and Methods) different volumes of the goat anti-ADH antiserum were added and immunoprecipitations were done as described in Materials and Methods. After immunoprecipitation, the ADH activity remaining in the supernatant was measured. As the volume of the antiserum was increased, the percent ADH activity remaining in the supernatant after immunoprecipitations decreased as expected. Thus, the antibodies do have the ability to precipitate the ADH protein.

The competition assays were done with 'op1 CyWT' (the complete genotypes are described in Materials and Methods), and 'op1 Cy;ry' extracts mixed with the wild type extract, and the results are shown in Fig. 25. As expected, when 50 µl and 100 µl of wild type extracts were used in immunoprecipitations, the 50 µl assay had approximately half as much activity remaining compared to the 100 µl assay. When 50 µl of wild type extract was mixed with 50 µl of 'op1 CyWT' (a strain carrying the opal suppressor tRNA) extract and assayed, there was no increase in ADH activity remaining in the supernatant. The same result was found with the 'op1 Cy;ry' and the wild type mixture. These results indicate no increase in ADH CRM^+ levels in the suppressor tRNA-carrying strains. This

Fig. 24 Graphical representation of the percent ADH activity remaining in the supernatant after immunoprecipitations with increasing volumes of anti ADH-antibodies. The total volume of each assay was kept constant (500µl) and each experimental assay contained 100 units (defined in Materials and Methods) of the wild type ADH activity (equivalent, in this case, to 50 µl of wild type extract).

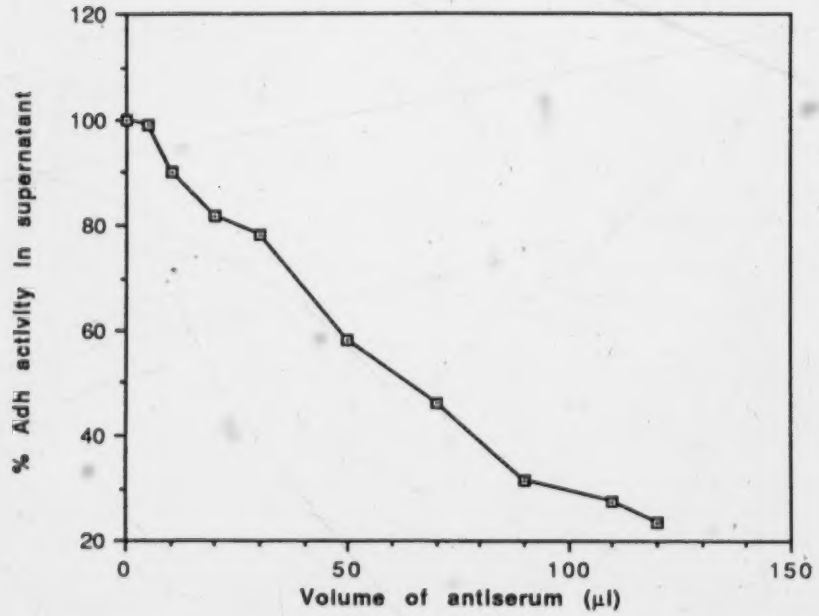

120
100
80
60
40
20
% Adh activity in supernatant
0
50
100
150
Volume of antiserum (µl)

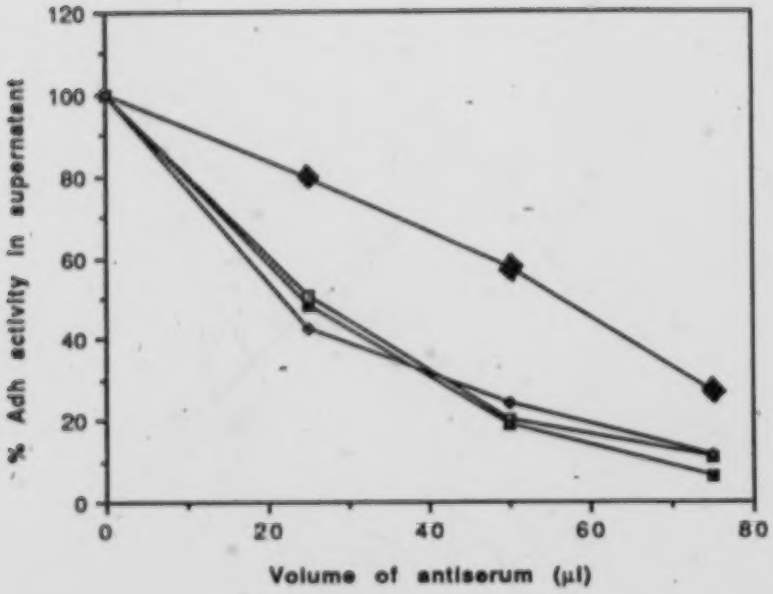
Fig. 25 Graphical representation of the results of the competition assays done with extracts from strains carrying the opal suppressor $tRNA^{Ser}$ gene ('op1 CyWT') and the extracts from a wild type strain.

◆ wild type strain (100 µl of extract);

▣ wild type strain (50 µl of extract);

■ 'op1 CyWT' and wild type extracts mixed (50 µl each)

◆ 'op1 Cy ry' and wild type extracts mixed (50 µl each).


120
100
80
60
40
20
0
% Adh activity in supernatent
0
20
40
60
80
Volume of antiserum (µl)

result is consistent with the conclusion that the CRM^+ levels are not augmented because no inactive (albeit full length) proteins are produced in the strains carrying the suppressor tRNA genes.

The graph in Fig. 26 indicates the results obtained in similar competition assays for 'am13 CyWT' (a strain carrying amber suppressor tRNA) and 'am13 Cy ry' extracts when they were mixed with wild type extracts. No significant differences were found between the CRM levels of the mixed extracts and the wild type extract alone, in this case either. Thus, the results obtained for both opal and amber suppressors indicate that there were no detectable levels of suppression in the presence of the appropriate nonsense suppressor tRNA gene.

16. Transcription studies

Since the competition assays did not reveal any measurable increase in ADH CRM levels, the next logical step was to estimate the transcription levels of these suppressor tRNAs in the strains carrying them. The serine suppressor tRNAs differ from the endogenous wild type $tRNA^{Ser}$ by only two bases in the anticodon. If the wobble capacity of the modified base (I) in the wild type anticodon is considered, the difference between the wild type and the suppressor tRNAs will only be one base. Since wild type tRNAs would be the most abundant species present in the tRNA preparations, the interpretations had to be based on differential Northern analyses.

The conditions of hybridization and washing, described in Materials and Methods, were optimized so that the suppressor tRNA could be distinguished from the endogenous wild type $tRNA^{Ser}$. A Northern blot of the tRNA isolated from the opal suppressor tRNA carrying strain (op1 CyWT) is shown in Fig. 27. The probe used was SPA#3 (Fig. 6) which specifically recognizes the opal suppressor $tRNA^{Ser}$ with the hybridization conditions used. In Fig. 27, lane 3 contains the tRNA from the strain, 'op1 CyWT' and there is no detectable signal in that lane. This indicates that the suppressor tRNA, if

Fig. 26 Graphical representation of the results of the competition assays done with extracts from strains carrying the amber suppressor $tRNA^{Ser}$ gene('am13 CyWT') and the extracts from wild type strain.

◆ wild type strain (100 µl of extract);

⊡ wild type extract (50 µl);

◆ 'am13 Cy ry' and wild type extracts mixed (50 µl each);

■ 'am13 CyWT' and wild type extracts mixed (50 µl each).

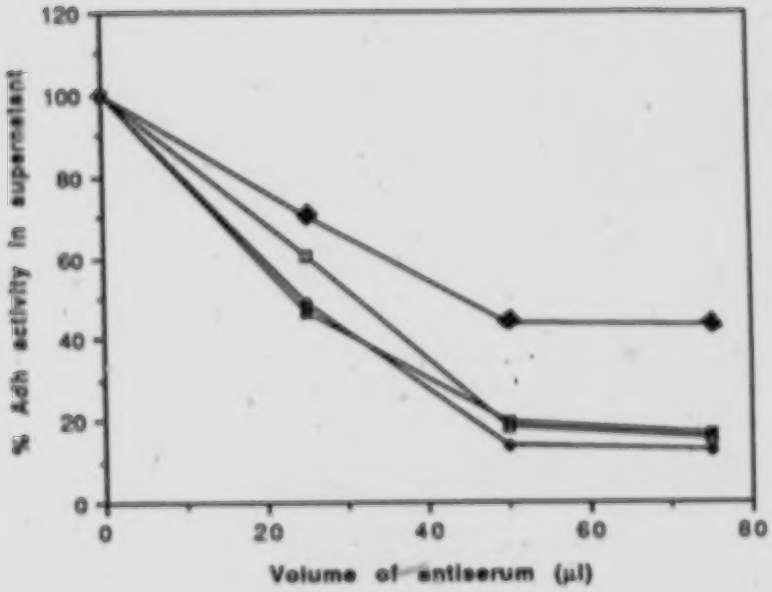

120
100
80
60
40
20
0
% Adh activity in supernatant
0
20
40
60
80
Volume of antiserum (µl)

Fig. 27 Northern hybridization analysis of tRNA fractionated from flies carrying the opal suppressor $tRNA^{Ser}$ gene. Thirty micrograms of total tRNA were loaded into each lane and electrophoresed on a 10% polyacrylamide, 8.3 M urea gel. The tRNAs were blotted onto a GeneScreen Plus membrane using a Bio-Rad Transblot cell, and probed with 5' end-labeled SPA#3 probe (with SPA#2 and 5 as cold competitior oligonucleotides, details in Fig. 6). The autoradiograph used for this photographic presentation was exposed for 24 hrs. The same result was seen even after an exposure time of 15 days.

Lane 1: tRNA from an untransformed yeast strain JG 369-3B(α) used as a negative control; Lane 2: *E. coli* tyrosine tRNA (1 μg) used as a size marker on the ethidium bromide stained gels; Lane 3: Transfer RNA from the 'op1 CyWT' strain carrying the opal suppressor tRNA gene; Lane 4: Transfer RNA from the 'op1 Cy ry' strain (without the suppressor tRNA gene); Lane 5: Transfer RNA from an untransformed fly strain; Lanes 6 and 7: Transfer RNAs from yeasts transformed with opal suppressor and wild type $tRNA^{Ser}$ genes of Drosophila, used as positive and negative controls respectively. The arrowhead indicates the expected size of the opal suppressor $tRNA^{Ser}$(85 nucleotides). The slightly smaller band in lanes 1, 6, and 7 represents cross hybridization to the yeast sequences (discussed in text), and is 76 nucleotides long.

1
2
3
4
5
6
7

transcribed, was transcribed at a level that was undetectable under the conditions used. The band shown with an arrowhead in lane 6 indicates the opal suppressor $tRNA^{Ser}$ from a yeast containing the opal suppressor gene (section 7 of this Chapter). Absence of a band of corresponding size in lane 7 (containing tRNA from yeast transformed with a wild type $tRNA^{Ser}$ gene) indicates that the hybridization and wash conditions were optimized for specific detection of only the suppressor tRNA. The 76 nucleotide-sized band seen in lanes 1, 6, and 7 indicate that the probe (SPA#3) used has homology with the yeast sequences. Comparison with published yeast tRNA sequences indicated that 15 out of the 16 bases in the oligonucleotide probe (SPA#3) are homologous to yeast $tRNA^{Arg}$. This analysis was also done with cold competitor oligonucleotides (SPA#2 and SPA#5 in Fig. 6), but no difference was found in the band pattern.

To ascertain the sensitivity associated with this lack of detectable transcription of the suppressor genes in Drosophila, another Northern analysis was done with the tRNA from yeast transformed with the opal suppressor $tRNA^{Ser}$ (pSS152) gene. The results are shown in Fig. 28, and as can be seen from the Northern blot, a titration of the opal $tRNA^{Ser}$ was made, in an attempt to determine the lowest amount of tRNA from the transformed yeast strain in which the opal suppressor $tRNA^{Ser}$ species could be detected. Lane 5 indicates that the lowest amount of yeast tRNA used, in which the opal suppressor $tRNA^{Ser}$ species is still detectable is 0.5 μg, under the hybridization and wash conditions used. In Fig. 27, this species is not detectable when 30 μg of Drosophila tRNA (from 'op1 CyWT') were used. This indicates that, if the opal suppressor $tRNA^{Ser}$ is transcribed in the 'op1 CyWT' strain, it is expressed at a level of less than 1.6% compared to the level that is sufficient for suppression in the *S. cerevisiae*.

Transcription levels were also studied for the amber suppressor tRNA gene in strain, 'am13 CyWT' (Materials and Methods) as shown in Fig. 29. As in the case of the opal suppressor tRNA, no detectable levels of expression were found for the amber suppressor $tRNA^{Ser}$ gene in strain 'am13 CyWT' as shown by the absence of a signal in

Fig. 28 Northern hybridization analysis of the tRNA from yeast transformed with the plasmid pSS152, which contains the opal suppressor $tRNA^{Ser}$ gene cloned into the YRp7 vector (details in Table 3).

Varying amounts of total tRNA were electrophoresed on a 10% acrylamide, 8.3 M urea gel. The tRNAs were blotted onto a GeneScreen Plus membrane and probed with SPA#1, to detect the Drosophila tRNA species. The exposure time used was 24hrs.

Lane 1: 30 μg of tRNA;

Lane 2: 10 μg of tRNA;

Lane 3: 3 μg of tRNA;

Lane 4: 1 μg of tRNA;

Lane 5: 0.5 μg of tRNA.

The arrowhead indicates the tRNA species of desired size (85 nucleotides).

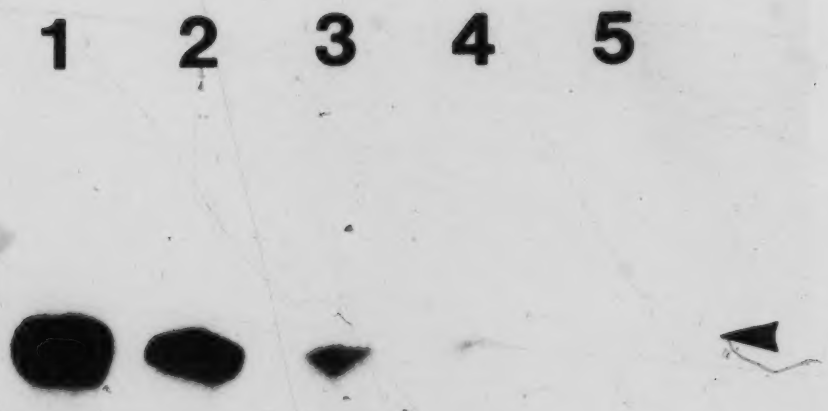

1 2 3 4 5

Fig. 29 Northern hybridization analysis of the tRNA fractionated from flies carrying the amber suppressor $tRNA^{Ser}$ gene.

Thirty micrograms of the total Drosophila tRNA per lane were electrophoresed in a 10% acrylamide, 8.3 M urea gel. The tRNAs were blotted onto a GeneScreen Plus membrane and probed with 5' end-labeled SPA#4 (with SPA#2 and SPA#5 as the cold competitor oligonucleotides (details in Fig. 6). The autoradiograph used for this photographic presentation was exposed for 24 hrs at -45° C. The same result was seen even after 15 days of exposure.

Lane 1: Transfer RNA from 'am13 CyWT', a strain carrying the amber suppressor $tRNA^{Ser}$ gene;

Lane 2: Transfer RNA from 'am13 Cy ry', a strain without the suppressor tRNA gene, (used as a negative control);

Lane 3: Transfer RNA from an untransformed fly strain (used as negative control);

Lanes 4 and 5: Transfer RNAs (10μg each), from yeasts transformed with amber suppressor and wild type $tRNA^{Ser}$ genes of Drosophila (used as positive and negative controls,respectively).

The arrowhead indicates the expected size (85 nucleotides) of amber suppressor $tRNA^{Ser}$.

1 2 3 4 5

lane 1. The only band that could be observed (Lane 4) corresponds to the amber suppressor $tRNA^{Ser}$ gene (pSS182) expressed in *S. cerevisiae*. Use of cold competitor oligonucleotides and/or less stringent conditions did not influence the band pattern observed.

A titration analysis of the amber suppressor $tRNA^{Ser}$ expressed in yeast is shown in Fig. 30, which allowed an estimation of the sensitivity of quantitation associated with the lack of expression of the amber $tRNA^{Ser}$ in the flies. Lane 5 contains the lowest amount (0.19 μg) of tRNA used in the analysis in which the amber suppressor $tRNA^{Ser}$ species was still detectable. This indicates that the level of the amber $tRNA^{Ser}$ in strain 'am13 CyWT', if it were transcribed, was less than 0.6% of the level that was sufficient for biological suppression in *S. cerevisiae*.

Fig. 30 Northern hybridization analysis of the tRNA from yeast transformed with the plasmid pSS182 (details in Table 3), containing an amber suppressor $tRNA^{Ser}$ gene of Drosophila.

Varying amounts of the total tRNA were electrophoresed in a 10% acrylamide, 8.3 M urea gel, blotted onto a GeneScreen Plus membrane and probed with SPA#1 to detect the Drosophila tRNA species. The exposure time used was 24 hrs at -45° C.

Lane 1: 15 μg of tRNA

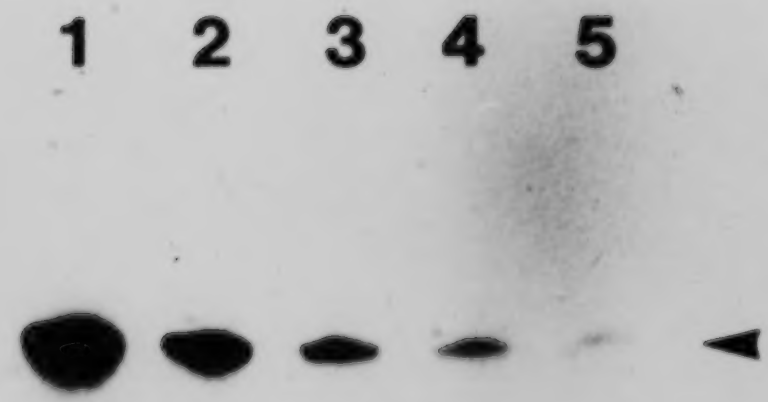
Lane 2: 5 μg of tRNA

Lane 3: 1.6 μg of tRNA

Lane 4: 0.5 μg of tRNA

Lane 5: 0.19 μg of tRNA

The arrowhead indicates the tRNA species of desired size.


1 2 3 4 5

Chapter IV

DISCUSSION AND CONCLUSIONS

Transfer RNA mediated nonsense suppression is the focus of this study. This type of suppression is mediated in many experimental systems by an altered anticodon (see Steege and Söll, 1979; Egel et al., 1980; Sherman, 1982; Hodgkin et al., 1987; Eggertsson and Söll, 1988 for reviews) capable of recognizing the appropriate nonsense codon and allowing the insertion of an amino acid at that position. Such nonsense suppressor tRNAs have been described in many organisms both prokaryotic and eukaryotic. These include *E. coli*, *S. cerevisiae*, *S. pombe*, Neurospora, *C. elegans*, mouse, rabbit, and cattle.

For many years identifying such nonsense suppressor tRNAs in Drosophila has been the main interest of our laboratory. In our laboratory, we attempted to identify second site revertants of a nonsense allele of Xdh (Girton et al.,1979) No candidates for nonsense suppressor tRNAs were identified in that screen although it produced revertants. In another laboratory a similar search was conducted among 21 genetically characterized suppressor stocks (Bienz and Kubli., 1981) and no suppressor tRNAs were identified. Later, a wild type Drosophila $tRNA^{Tyr}$ (with an anticodon GψA) was shown to suppress a leaky UAG codon of the TMV RNA in Xenopus oocytes (Bienz and Kubli, 1981). However, efforts to identify suppressor activity in the tRNA fractions of those suppressor stocks proved unsuccessful.

The advent of the technique of P-element mediated germline transformation of Drosophila (Rubin and Spradling, 1982; Spradling and Rubin, 1982) facilitated the introduction of extant suppressor tRNA genes into Drosophila. Many yeast suppressors (from *S. cerevisiae* and *S. pombe*) have already been identified or synthesized and also cloned (see Egel et al., 1980; Sherman, 1982 for reviews, Hottinger et al., 1982; Hottinger et al., 1984; Sumner-Smith et al., 1984; Krupp et al., 1985). It has also been

demonstrated that some of the suppressor tRNA genes are active in a heterologous mileau (Hottinger et al., 1982; Hottinger et al., 1984; Sumner-Smith et al., 1984). There is evidence indicating that transcriptional and processing activities are sufficiently well conserved among eukaryotic tRNA genes (see Sharp et al., 1985 for a review) such that *in vitro* and *in vivo* expression of heterologous tRNA genes is often possible.

Therefore, the emphasis in our laboratory switched from attempts to induce an *in vivo* suppressor by mutagenesis towards introducing extant suppressor tRNA genes into Drosophila by transformation. Previous work (Molnar et al., 1988) involved the transformation of *D. melanogaster* with the Sup3e tRNA gene (Hottinger et al., 1982) from *S. pombe*. This tRNA inserts serine in response to the UGA nonsense codon in *S. pombe*. When transformants were obtained, they were crossed into a genetic background containing an *Adh*⁻ nonsense mutation (Adh^{nB}), in which tryptophan at position 234 was changed to a UGA codon (Benyajati et al., 1981; Kubli et al., 1982). When assays for ADH activity were done, no activity was found. When the present project was started we thought that the above transformants did not show any ADH activity for one of two reasons: firstly, Sup3e, being a heterologous gene, was not expressed or processed well by the Drosophila transcription machinery; or secondly, the amino acid inserted by Sup3e (serine) tRNA was not appropriate at that position of the protein, leading to an enzymatically inactive product.

This project was started with an objective of finding which one of these hypotheses was true. Since the *S. pombe* gene did not function in Drosophila (Molnar, 1985) we decided to see if Drosophila suppressor tRNA genes would show activity in a homologous system. We chose $tRNA^{Ser}_{7}$ gene from *Drosophila melanogaster* for this study (Dunn et al., 1979; Cribbs et al., 1987a; 1987b). This gene, being of homologous origin, would help us determine if serine was inappropriate at that position of the ADH protein since expression and processing would not be expected to pose a problem. This objective was made feasible by the recent advances in *in vitro* mutagenesis techniques (Zoller and Smith,

1982; Kunkel, 1985; Norris et al., 1983) which allowed *in vitro* contruction of the suppressor tRNA genes from wild type Drosophila tRNA genes. Further, the development of P element-mediated germ line transformation techniques in Drosophila allowed the reintroduction of these engineered tRNAs into flies.

A clone of the $tRNA^{Ser}_{7}$ gene was obtained from Dr. G.M. Tener, University of British Columbia, Vancouver. A 2.4 kb *Pst*I-*Hin*dIII fragment (Fig. 5) from this clone was sub-cloned into the M13mp18 phage vector. This recombinant derivative (M-PH1) was utilized as the substrate for oligonucleotide-directed, site-specific mutagenesis (Fig. 7) (Zoller and Smith, 1982; Kunkel, 1985). Using the oligonucleotides indicated in Fig. 6, an array of all three (opal, ochre and amber) nonsense suppressor tRNA derivatives were constructed (Fig. 8). Theoretically, if deoxyuridine were incorporated into all the single stranded M13 templates and used in site-specific mutagenesis, the recovery of mutants should be at 100% efficiency. In my hands, the efficiency was found to be 10-20%, which may reflect the efficiency with which uridine is incorporated into the template DNA in the strain BW313 (dut^- ung^-). An upstream oligonucleotide was used as a second primer in each mutagenesis to aid in stable annealing of the mutant oligonucleotide to the target site as suggested by Norris et al. (1983).

It has been previously reported that some tRNA genes exhibit *in vitro* and *in vivo* transcription, processing and biological activity in a heterologous mileau (Hottinger et al., 1982; Laski et al., 1984; Capone et al., 1985). The promoter sequences of the eukaryotic tRNA genes are relatively highly conserved (Sharp et al., 1985; Sprinzl et al., 1989), and this would account for the ability of heterologous tRNA genes to be transcribed. Apparently, the machinery for many of the subsequent events such as processing and modification must also be sufficiently conserved so as to permit some heterologous tRNA genes to be productively expressed. But this is not always the case, since it was found that the *S. pombe* suppressor genes function with strict codon-specificity when introduced into the genome of *S. cerevisiae* (Hottinger et al., 1982; 1984), but the reverse was not found to

be true (Krupp et al., 1985). Also, an *S. pombe* $tRNA^{Ser}$ gene was found to be non-functional in Drosophila (Molnar et al., 1988) but was functional when in *S. cerevisiae*.

In some heterologous systems suppressor tRNA genes lose their codon specificity (Rossi et al., 1982). When an *S. cerevisiae* ochre suppressor $tRNA^{Tyr}$ was introduced into *E. coli* it was shown to suppress both ochre and amber nonsense mutations. This pattern of suppression, i.e., suppression of both ochre and amber nonsense mutations, is characteristic of *E. coli* ochre suppressor tRNAs and is in contrast to the pattern observed in *S. cerevisiae*, where only ochre mutations are suppressed. The codon specificity is retained even when heterologous suppressors are introduced into *S. cerevisiae* (Hottinger et al.,1982; 1984). Therefore, we decided to first test our Drosophila suppressor constructs in a heterologous system to study their activity and codon specificity. *S. cerevisiae* was chosen as the heterologous system in which these constructs were to be tested. For this purpose, the wild type $tRNA^{Ser}$ gene and the three mutant constructs were cloned into an *E. coli*-yeast shuttle vector, YRp7 (Fig. 10). The recombinant plasmids carrying the wild type gene (pSS166) or the opal (pSS152), ochre (pSS179) and amber (pSS182) suppressor derivatives were then used to transform *S. cerevisiae*.

The host strain of yeast that was used as the recipient in these transformation experiments was JG 369-3B (α). This strain contains an array of nonsense mutations (Table 3), and is also easy to transform (J.P. Gelugne, personal communication). These features made it suitable for the *in vivo* suppression assays. The results of the *in vivo* assays were presented in Table 3. All three nonsense suppressor derivatives of Drosophila were found to be biologically active in *S. cerevisiae*. This is an independent demonstration that the *in vitro* mutagenesis of the Drosophila tRNA genes produced an active suppressor. The codon specificity of all the suppressor genes was also retained. The only nonsense mutations that were exceptions to the above were *ade*2-1 and *can*1-100. The former was poorly suppressed while the latter was not suppressed at all by the Drosophila suppressor genes. The non-suppression of the *can*1-100 marker by the Drosophila $tRNA^{Ser}$ gene is in

agreement with the homologous yeast suppressor results found by others (J.P. Gelugne, personal communication; Ono et al., 1979). In the latter work, class III suppressors of yeast which insert serine were found not to suppress the UAA mutation of *can*1-100, but found to suppress other UAA mutations.

For *ade*2-1, we found very low efficiency of suppression. The criteria for suppression in this case were both change of color and growth. Growth was noticed only after 5-7 days of incubation at 30° C, while there was slight color change from red to pale pink. Similar results were reported for SUP-17 in yeast, also a serine inserting UAA suppressor (Ono et al.,1979). This suppressor was shown to suppress the auxotrophic requirement of adenine for growth but the transformants were pink, as is in our case. The ability of the Drosophila genes to function in *S. cerevisiae* indicates that the 2.1 kb fragment containing the Drosophila $tRNA^{Ser}$ gene (wild type or mutant form of the gene) contains all sequences necessary for their recognition and transcription by the yeast RNA polymerase-III and other transcription factors. Once a Drosophila tRNA gene is transcribed, it is treated like an endogenous transcript since processing and aminoacylation also take place to produce an active suppressor. Of course, I have not been able to see if serine is actually inserted in response to the yeast nonsense codon. However, it can be ascertained with certainity that 'some' amino acid is inserted, which restores biological function to the 'suppressed' protein.

Initially, the most logical sequence to define the identity of a tRNA was thought to be the anticodon (reviewed in Kisselev, 1985). Work done by several groups was in agreement with this idea and the anticodon was an essential identity element for $tRNA^{Met}$, $tRNA^{Val}$ (Schulman and Pelka, 1988), $tRNA^{Gln}$ (Yaniv et al., 1974), and $tRNA^{Phe}$ (Sampson and Uhlenbeck, 1988). However, this was not the case for all the tRNAs (see Yarus, 1988; Schulman and Abelson, 1988 for reviews), and $tRNA^{Ser}$ is one of this latter group. In $tRNA^{Ser}$ of *E. coli*, nine nucleotides were found to comprise the identity set (Normanly et al., 1986a; Schulman and Abelson, 1988). These include the three base pairs

at the end of the acceptor stem, G73, and C11-G24 (a base pair in the D stem). These findings allow me to speculate that the base changes made in the anticodon of the Drosophila $tRNA^{Ser}$ gene did not change the identity of the transcripts, and that the suppression found in the yeast, *S. cerevisiae* is likely due to the insertion of serine in response to the appropriate nonsense codon. In addition, the -5 to -20 region implicated by St. Louis and Spiegelman (1985) and Cribbs et al. (1987a) in positively modulating the expression of this $tRNA^{Ser}$ gene of Drosophila is also present in our constructs.

To ascertain the levels of transcription and the accuracy of processing of these Drosophila suppressor tRNA genes in *S. cerevisiae*, Northern hybridization analyses were done. As shown in Fig.11, all the constructs including the wild type gene were found to be transcribed and processed well, producing a tRNA species of the expected size. To verify the association of these tRNA species with the presence of the plasmid, the transformant yeast strains were grown on complete medium (YEPD). Since these constructs are all derivatives of YRp7, which is a replicating yeast plasmid, these will be lost from the cells in the absence of any selection. The strains that lost the plasmid (identified by their inability to grow on tryptophan omission medium) were assayed for suppression, and it was found that all the strains, without exception, also lost their ability to exhibit any suppressor phenotype. Transfer RNAs were then prepared from the strains that lost their ability to suppress and were assayed on a Northern blot (Fig. 12). No detectable signal corresponding to the Drosophila tRNA species was found in any of the lanes. The results obtained from this Northern analysis (Fig. 12), confirmed that the tRNA species present in the 'suppressed' strains were specifically associated with the genes present on the replicating plasmid.

A comparison of the box A (+8 to +30) and box B (+51 to +72) (Lewin, 1987; Sprinzl et al., 1989) sequences of Drosophila and yeast $tRNA^{Ser}$ genes revealed that these regions are well conserved between these two organisms. There is a 87-91% homology in the box A region and 71-76% homology in the box B region, which apparently is sufficient

for the transcription of these Drosophila tRNA genes by the yeast machinery. However, Fig. 11 indicates that these different derivatives of the $tRNA^{Ser}$ gene of *D. melanogaster* are transcribed to varying levels by the yeast machinery, the least expressed species being the opal $tRNA^{Ser}$ and the most expressed species being the wild type tRNA . This indicates that the base changes, introduced into the anticodon, do have an effect on the level of transcription, although they do not completely abolish it. Since no other changes were found in the suppressor tRNA genes when compared to the wild type sequence (as revealed by DNA sequence analysis), any effects on transcription levels would be expected to be due to the changes introduced into the anticodon.

My results also indicate that the wild type anticodon sequence is not absolutely essential for the transcription and processing of this $tRNA^{Ser}$ species of *D. melanogaster*, at least in this specific heterologous milieu. The varying levels of transcription of these suppressor tRNA genes which are all biologically active, also indicate that expression at the wild type level is not required for efficient suppression.

The normal growth of transformant colonies within 2-3 days indicates that there are no strong adverse effects of these heterologous suppressor tRNAs on the translation machinery. That is, read through translation of the other mRNA transcripts does not seem to be occurring at intolerable levels. The only problem that was noticed was with the amber suppressor $tRNA^{Ser}$ construct. When tRNA was purified from yeast transformed with the amber suppressor $tRNA^{Ser}$ and analysed on a Northern blot it was found that the amber suppressor carrying plasmid (pSS182) was lost from the yeast cells 50% of the time (data not shown). This finding is in agreement with other reports where strong nonsense suppressors were found to have adverse effects. Strong suppressors in *S. cerevisiae* are reported to be always associated with slow or impaired growth (see Sherman, 1982 for a review) and, more specifically, it is the amber suppressors that have the most deleterious effects on yeast cell growth (Liebman and Sherman, 1976). Another example of an organism being sensitive to suppressor tRNAs is found in *C. elegans*. The strong amber

suppressor sup-7 (Waterston, 1981) leads to sterility or lethality in homozygous animals grown at low temperature.

The above analyses done in a heterologous system indicated that the base changes made in the constructs, by themselves, were not inactivating expression of the suppressor tRNA genes. Therefore, the next thing to be done was to study the expression of these *in vitro* constructed suppressor tRNA genes in the homologous Drosophila system.

For this purpose, all the constructs were subcloned into the *E. coli*-Drosophila shuttle vector, Carnegie20 (Figs 13 and 14). The recombinant plasmids containing the wild type $tRNA^{Ser}$ gene (pSS193), or the opal (pSS06), ochre (pSS11f) or amber (pSS182) derivatives were used to perform germline transformations of the appropriate Drosophila strain. The Drosophila strain used in all the transformation experiments was *b cn;ry*^{8} (the complete description of the genotype is given in Materials and Methods). The reason for using this strain in the transformations, instead of the strains carrying the appropriate nonsense mutation, was that it was previously found in our laboratory (Molnar, 1988) that this strain is easier to transform.

P element-mediated germline transformations (Rubin and Spradling, 1982; Spradling and Rubin, 1982) were used to introduce the suppressor tRNA gene constructs into the genome of Drosophila. The strategy of this technique was presented in Fig. 15. The pπ25.7wc plasmid (Karess and Rubin, 1984) was used as a helper in the transformations. The P elements in the Carnegie20 constructs were not complete and also were crippled due to the insertion of the rosy gene and the tRNA gene constructs between the two ends of the P element. Therefore, these P elements were not autonomous and cannot transpose into the genome of Drosophila, because they lack an ability to produce transposase. The helper plasmid, pπ25.7wc serves the purpose of providing the transposase. Another advantage of using pπ25.7wc as a helper, instead of other available helper plasmids (like pπ25.1), is that in the former one of the inverted repeat sequences at the ends of the P-element is not intact, thereby preventing the integration of the helper

plasmid itself. This leads to increased stability of the integrated Carnegie20 constructs due to lack of transposase in subsequent generations.

Microinjections of embryos were initially done using chemical dechorionation. This hastens the processing and aligning of the embryos for injections and helps in preventing the over-desiccation of embryos before injections. However, we found that chemical dechorionation led to a reduced survival to hatching. This finding was supported by the report of Chou et al. (1987) which indicated that the bleach used to dechorionate the embryos selectively kills the embryos at the stage when the P elements transpose. Therefore, all the injections in the later stages of this work were done using mechanical dechorionation of the embryos under high humidity condtions (70-80%). Under these conditions, the time required for sufficient desiccation of the dechorionated embryos was found to be about 25-30 minutes. The area where the injections were done was also kept at a temperature of 18° C to reduce the shock of injections to the embryos. After injections, the embryos were kept at the same temperature (18° C) until hatching, to get better recovery. All these measures increased the rate of survival to hatching from 10% to about 30-50%.

As indicated in Fig. 15, all the integration events were kept separate by independently crossing each G_0 fly and each G_1 ry^+ fly to flies with the untransformed genotype (*b cn;ry*^{8}). The $tRNA_7^{Ser}$ gene normally produces one of the major $tRNA^{Ser}$ species (White et al., 1975) among all reported serine tRNA genes. Therefore, we thought that if transposons with integrations at different locations within the Drosophila genome were brought together by intercrossing in the G_0 and/or G_1 generations, this might lead to increased expression of the suppressor tRNA genes leading in turn to intolerance by the flies resulting in sterility and reduced survival. So, the transformed lines were maintained by intercrossing only after the flies had survived to G_2 generation (by back-crossing to flies of the untransformed genotype).

The transformed lines were all derived from the *b cn;ry*^{8} strain, which carried only wild type alleles of the *Adh* locus. Therefore, all the suppressor tRNA genes were brought into an appropriate genetic background containing a nonsense mutation, by the genetic crosses schematically represented in Figs. 16 and 17. The reason for using the two types of crosses was presented in the Results section. In addition to serving the purpose of bringing the suppressor tRNA genes into the appropriate genetic background, one of these crosses (Fig. 16) also helped in establishing which chromosome carried these new suppressor tRNA genes. The results of this analysis were presented in Table 9, and the logic followed in assigning the transposons to one of the chromosomes is discussed in the Results section. As mentioned therein, these crosses would not permit testing the integration events on the second chromosome for suppressor activity of the tRNA gene constructs.

The chromosomal locations for some of the lines were also determined by *in situ* hybridizations to the salivary gland chromosomes. In doing these hybridizations, both glands of a pair of the salivaries were squashed. One of each pair was probed with the tRNA probe and the other with the rosy probe. This unequivocally established the location of the transposon in each case. Since the transposon contained both the rosy gene and the tRNA gene, both probes recognized one common location as expected. This result confirmed that each transposon selected on the basis of ry^{+} phenotype still contained the 2.1 kb fragment that contained the tRNA^{Ser} gene.

When the ADH assays were done as previously described, no ADH activity was found. Initially this could have been due to any of the following reasons: (a) the ry^{+} lines selected were not real transformants but the ry^{+} phenotype was observed due to a second site mutation, (b) the base changes introduced into the tRNA gene have an effect on transcription, or that the constructs did not have the sequences required for efficient expression, (c) the suppressor tRNA gene was not tolerated by the flies, leading to its deletion, (d) serine was incorporated into the protein, but no activity was seen because

serine is not appropriate at that position of the protein, (e) there was insufficient expression of the suppressor tRNA leading to undetectable levels of expression, or (f) suppression is allele-specific, such that only nonsense mutations in certain loci could be suppressed by a specific suppressor tRNA.

The genomic Southern analysis presented in Fig. 18 argues against the lines not being real transformants. When the genomic DNA from the putative transformants was probed with the radiolabeled 2.1 kb fragment containing the $tRNA^{Ser}$ gene, an additional band was seen in all the lanes containing the DNA from these transformants, which was not present in the untransformed control lane. Also, the data from the *in situ* hybridization analyses support the conclusion that all transformants contain the suppressor tRNA gene.

The *in vivo* assays done in *S. cerevisiae* indicated that the base changes introduced into the Drosophila $tRNA^{Ser}$ gene did have an effect on the level of transcription, in some cases reducing the level quite drastically (in the case of opal suppressor tRNA, for instance). All of the suppressor constructs exhibited a reduction in the level of transcription, compared to the levels of the unmutated wild type $tRNA^{Ser}$ gene. However, as was revealed in Fig. 11, processing and maturation of tRNA were not affected. A tRNA species of the expected size was found in all of the lanes containing tRNA from yeast transformed with the suppressor tRNA genes. Although the base changes resulted in reduced levels of expression of the Drosophila tRNAs, the levels were found to be sufficient for suppression in yeast. Furthermore, the Northern analyses detected no evidence of accumulation of any processing intermediates, which suggests that all heterologous transcripts produced were accurately and completely processed.

The genomic Southern analysis presented in Fig. 23 was aimed at determining if the suppressor tRNA gene was deleted in any of the transformed lines. As can be seen in that figure, when the genomic DNA was probed with an oligonucleotide (SPA#1) that recognized only the tRNA gene sequences an extra band was seen in each of the

transformed lines which was not present in the untransformed control lanes. This argues against any deletion of the suppressor tRNA gene component of the integrated transposons.

Therefore, it seemed that the most likely remaining explanation for the failure to observe suppression was that serine is an inappropriate substitute for tryptophan at that position, leading to the production of a biologically inactive protein. The occurrence of a nonsense mutation in an enzyme-coding gene often leads to loss of both enzymatic activity and immunologically cross-reacting material (CRM) (Weigert and Garen, 1965). Therefore, in the strains carrying nonsense mutations for *Adh*, ADH CRM levels should be low or absent. For the example of the CyO^{nB} allele used in the present study, varying levels of CRM have been reported. In one case the Adh^{nB} mutant was found to have no detectable CRM levels (Schwartz and Sofer, 1976), while other reports indicated this mutant to be CRM positive (Martin et al., 1981). For the Adh^{n4} mutation cross reacting material was not reported (Chia et al., 1987). If serine, though inappropriate at that position, was incorporated into the protein, it should lead to the production of an enzymatically inactive full-length protein. This protein would be expected to increase the amount of CRM bound by anti-ADH antibodies. The inactive 'suppressed' protein should compete with the wild type ADH protein for the antibodies. However, as was displayed in the figures 25 and 26, no such competition could be detected with extracts from lines harboring either the opal or the amber suppressor tRNA. This indicated that there was so little suppression it was undetectable or that there was no suppresison at all. Thus, I was unable to determine if serine is an appropriate amino acid or not.

Very low levels of transcription of the serine tRNA could also result in lack of suppression. Initially, this did not seem likely since the experiments put a Drosophila gene back into Drosophila. One would not, *a priori*, expect any transcriptional, processing or aminoacylation problems with a completely homologous system. However, we decided to study the copy number and the transcriptional levels of the transposons in the opal and amber transformant strains.

The genomic Southern analysis presented in Fig. 19 was done with the objective of finding out the copy number of the suppressor tRNA genes per transformed line. As is clear from that figure, there is only one copy of the transposon per transformed line. The pπ25.7wc plasmid was used as the helper in the transformations. Since it can not integrate by itself, the availability of transposase was limited to a short period of time. This might allow one to conclude that this could be the reason for the low copy number. But, work done in our laboratory and other reports (Klemenz et al., 1987) indicate that it was frequently possible to obtain more than one copy of the transposon per transformed line. Thus, getting only one copy in all of the analyzed transformed lines reported herein might not be just a coincidence.

The transcription studies done are presented in Figs. 27 and 29. As detailed in the results section, there were no detectable levels of expression of the suppressor tRNA in either the opal or the amber suppressor-carrying lines. The suppressor tRNA species were not detectable even after two weeks of exposure. Figures 28 and 30 represent the Northern blots of the opal and the amber suppressor $tRNA^{Ser}$ species of Drosophila in yeast. In these two experiments, an effort was made to determine a minimum amount of total yeast tRNA in which the Drosophila suppressor tRNA species were still detectable. This allows one to draw a comparison between the levels of the Drosophila suppressor tRNA species when expressed in yeast compared to when they are expressed in Drosophila. However, the suppressor tRNAs were not detectable in the latter. Therefore, using the data from Figs. 28 and 30, one can ascertain limits for the maximum levels of expression of suppressor tRNA genes in Drosophila. From such a comparison, it was concluded that the opal suppressor $tRNA^{Ser}$, if expressed in Drosophila, was expressed at less than 1.6% of the expression of the same gene when in yeast, and that the amber suppressor $tRNA^{Ser}$ was expressed at less than 0.6% of the level of the amber suppressor in yeast. The expression of opal and amber $tRNA^{Ser}$ genes in yeast were used to draw such a comparison instead of the wild type serine tRNA gene. This was done because in yeast the levels of the

Drosophila suppressor $tRNA^{Ser}$, although expressed at lower levels than the wild type $tRNA^{Ser}$ gene, were quite sufficient for suppression. This observation confirms that levels of suppressor tRNA comparable to wild type level are not required for suppression.

The above analysis indicated that the low levels of the transcription of these suppressor tRNA genes within Drosophila was the basis for the lack of suppression of the *Adh*^{n} nonsense mutations in Drosophila. If poor transcription is the problem, the most obvious explanation is that it was due to a position effect on tRNA gene expression. Poor transcription is the result of the position at which the transposons integrated into the Drosophila genome.

Suppressor tRNA genes can have a negative effect on the organism, since they are capable of read-through translation. This problem may be more effectively circumvented in other organisms by having strict codon context requirements for proper termination, so that the release factors outcompete the suppressor tRNAs at the natural termination sites (see Steege and Soll, 1979; Bossi and Roth, 1980 for reviews). The result would then be that higher levels of informational suppression would be tolerated in these organisms. It has previously been shown (Beaudet and Caskey, 1970; Ganoza and Tomkins, 1970) that the ratio of suppressor tRNA to release factors determines the ratio of readthrough translation to proper chain termination *in vitro*. In addition, several reports lent support to the idea of the influence of neighboring nucleotides on the recognition of termination codons (Salser, 1969; Salser et al., 1969; Yahata et al., 1970; Comer et al., 1974; Comer et al., 1975; Colby et al., 1976; Akaboshi et al., 1976; Fluck et al., 1977; Feinstein and Altman, 1977; Feinstein and Altman, 1978). A recent example of this kind was provided by Martin et al. (1988) who showed that the activity of the release factors varies according to the messenger context. It was demonstrated in their work that contexts which dictate a weak affinity for release factor will favor suppressor tRNA binding rather than termination and that nonsense mutations at those sites will therefore be suppressed more efficiently. In light of these

reports, it appears that a combination of different variables is required for efficient suppression and/or proper termination at the natural termination sites.

If suppressors are poorly tolerated in Drosophila we may have inadvertantly selected for those transformants in which the suppressor gene is poorly expressed. This is supported by the observation that all the transformants recovered during this work and previously in our laboratory are quite healthy and fertile. This is at odds with the findings of others (Doerig et al., 1988; Laski et al.,1989; D. Garza and D. Hartl, personal communication). In their work, all the transformants were found to be very weak with high levels of sterility, and could not be maintained beyond the G_1 generation. We also found some sterile tranformants that could not be maintained beyond the G_1 generation. However, most of the transformants were very healthy. A reasonable explanation for this finding could be that, as mentioned above, only those transformants with no, or very low, suppressor tRNA expression levels were recovered.

Another possibility that can be considered is the idea of repression, by the repressor of the P-element (see Engels,1988 for a review), of the expression of the genes present or inserted between the two P-element termini. This could explain the negligible levels of expression of the suppressor $tRNA^{Sup}$ gene, as measured on the Northern blots. However, two observations argue against P element repression. Firstly, when genomic DNA from the untransformed host strain was searched for autonomous P-element sequences using a probe that recognized the internal sequences of the P-element no such elements were found (data not shown). Secondly, all the Carnegie20 constructs used in the transformation experiments also contain sequences from the structural locus for Xanthine dehydrogenase (referred to as the 'rosy' gene throughout this thesis). The selection strategy used for identifying the transformants was to look for rosy locus expression in the putative transformants that were rosy$^-$. This ensured that the rosy locus was expressed in the transformants and that there was no interference with its expression. Both these observations argue against any repression of the constructs by the repressor of P-element.

Therefore, as mentioned above, the low level of expression of the suppressor tRNA gene is most likely due to its position in the genome such that only integrations leading to very low transcriptional activity could be recovered.

While work on this project was in progress, two groups reported obtaining very low levels of suppression in *D. melanogaster*. The work of Doerig et al. (1988) was done on the 'rosy' locus of Drosophila. Different rosy mutant stocks were injected with amber and ochre suppressor $tRNA^{Tyr}$ genes in an effort to identify nonsense mutations in that locus. Their work revealed that the $rosy^{516}$ mutant is an amber mutant. Six G_1 flies were found that exhibited an eye color intermediate between rosy and the wild type eye color. None of these G_1 flies could be propagated due to sterility; therefore, no further work could be done on these transformed flies. For the rosy locus, it has previously been suggested that less than 1% XDH activity is sufficient to produce normal or near-normal eye color (Chovnick et al., 1977; Girton et al., 1979). Therefore, the recovery of flies exhibiting an intermediate eye color (Doerig et al.,1988) indicates that the suppression levels were extremely low, and even then led to sterility and reduced survival. This further supports the speculation made earlier that suppressor tRNAs are not tolerated very well by flies. The tyrosine tRNA gene used to construct the suppressor mutants above (Doerig et al., 1988) would have a modified nucleoside in the anticodon (GψA). This nucleoside was suggested to be crucial for the efficiency of suppression in yeast (Johnson and Abelson, 1983). Therefore, it was not changed when base changes were introduced to construct the suppressor mutant tRNAs. In the present work the $tRNA^{Ser}$ gene used produces a product which normally has a modified nucleoside in the wobble position of the anticodon (IGA). This residue position had to be changed during the *in vitro* mutagenesis in the creation of all three nonsense suppressor genes. The results obtained in the yeast system indicate that the changes introduced did not completely abolish transcription of the mutant tRNA genes. However, the levels of expression varied between the mutant species, and all were expressed at lower levels than an unaltered wild type gene. Therefore, eliminating the

normally modified base could not be the sole reason for the lack of suppression in this case. Of course, this conclusion is based on the assumption that identical sequences are required for the expression of the suppressor tRNA gene in both yeast and Drosophila. Also, the varying levels of expression of these Drosophila suppressor genes in *S. cerevisiae* might simply be reflecting the effect of being in a heterologous system. In addition, reports indicate the identity set of the bases for serine tRNA does not include the bases in the anticodon (Normanly et al., 1986; Schulman and Abelson, 1988). Therefore, it is expected that both the wild type and the mutant tRNAs will be aminoacylated correctly and charged with serine.

Another group has also demonstrated suppression in *D. melanogaster* (Laski et al., 1989). In their work an amber suppressor $tRNA^{Tyr}$ of Drosophila was shown to suppress an amber mutation in the chloramphenicol acetyl transferase (CAT) gene of Tn9 when both the genes were introduced into Drosophila. The amber mutant [*CAT am(33)*] used in this work retained 0.1% of the CAT activity typical of the wild type gene. In the presence of suppressor tRNA, this level increased to 0.4% of the wild type level. The amber mutant *CAT am(33)* used was the result of a mutation of a tyrosine codon at position 33 to the amber codon. Since the suppressor tRNA was inserting the original amino acid wild type CAT protein should be produced. The low levels of CAT activity observed indicate that, as in our case, the suppressor tRNA is poorly expressed or context effects prevent efficient suppression. The same suppressor $tRNA^{Tyr}$ was tested for its activity against a second CAT amber mutant [*CAT am(27)*] in which a serine codon at position 27 was changed to an amber codon. In this case, no CAT activity was detectable, when using the suppressor $tRNA^{Tyr}$ of Drosophila. In yet another experiment, they tried to suppress an amber mutation in a P element, where a tyrosine codon was changed to an amber codon at position 2340. In this case also, no suppression was found.

In both *CAT am(33)* and the P element amber mutant the change was a tyrosine codon to the amber codon. When suppressor $tRNA^{Tyr}$ was used, suppression was

demonstrated in one case (*CAT am(53)* but not in the other (P element mutant). This could be due to the varying positions of the amber mutations within the two genes. Probably, at one location the nonsense mutation was easier to suppress than the other. Also, a higher level of product might be necessary to detect suppression in the case of the P element. In light of these results it would be interesting to try suppressing the *CAT am(27)* allele with our $tRNA^{Ser}$ amber suppressor since suppression would restore the wild type serine residue at position 27. This would be expected to result in CAT activity and the CAT assay is sensitive enough to detect even very low levels of suppression.

In conclusion, the Drosophila $tRNA^{Ser}$ gene used herein and its opal, ochre and amber suppressor derivatives were transcribed, processed and expressed in *S. cerevisiae*. The high level of sequence homology within the coding sequences of these tRNA genes (especially box A and box B regions) seems to be sufficient for the expression of these heterologous genes in the yeast background. Thus, *S. cerevisiae* is a suitable system for testing the function of heterologous tRNA suppressor genes.

The results obtained in the yeast system indicate that although the base changes introduced into the anticodon reduced the levels of transcription, significant expression was still obtained. This indicates that the wild type anticodon sequence is not an indispensable requirement for the expression of these serine tRNA genes.

In Drosophila, when our suppressor $tRNA^{Ser}$ genes were tested no biological suppression could be detected. Thus, it can be concluded that the presence of the suppressor tRNA gene is not lethal to the organism, but perhaps that the only transformants that could be recovered were those transcribed so poorly that they do not pose any threat to the survival of the organism. Genomic Southern analyses indicated that the suppressor tRNA gene was not deleted in the transformed flies. The levels of the suppressor tRNAs in Drosophila are less than 0.6-1.6% of the levels of the same tRNAs expressed in the yeast system. These low levels of expression are likely the main reason for the lack of suppression in this study. The work of others (Doerig et al., 1988; Laski et al., 1989)

indicates that the suppression in Drosophila is allele- and position-specific. These results indicate that it will be very difficult to suppress nonsense mutations in Drosophila *in vivo*.

To ascertain if our conclusions and speculations are valid, various experiments could be done to extend this study. *In vitro* translations can be done in the appropriate cell-free extracts to see if higher levels of suppressor tRNA would result in suppression. The messenger RNA could be isolated from the appropriate nonsense mutant of Drosophila (Adh^{nB} or Adh^{n4}, in this case), followed by *in vitro* translation of the message in a cell-free extract, in the presence of increasing amounts of the suppressor $tRNA^{Ser}$ (opal or amber). The source of the opal or amber suppressor $tRNA^{Ser}$ for such an experiment would be the corresponding Drosophila $tRNA^{Ser}$ mutants expressed in *S. cerevisiae*. The truncated protein in the nonsense mutant could be distinguished from the wild type protein by the difference in size. Therefore, if the translation products were immuno-precipitated in the presence of anti-ADH antibodies and electrophoresed on an SDS-PAGE gel, the amount of suppressor tRNA required for suppression could be determined.

A more sensitive assay for supression exploits the rosy system. As mentioned earlier, less than 1% XDH activity is sufficient to restore wild type eye color to a rosy homozygote. The suppressor constructs would be re-cloned into an appropriate vector like pUChsneo (Steller and Pirrotta, 1985) which has neomycin resistance as the selectable marker. Those constructs would then be tested against the array of nonsense mutations available in the rosy locus (Chovnick et al., personal communication; Doerig et al., 1988) by germline transformations of Drosophila. Such experiments have been tried with our constructs (data not shown) using the pW8 vector (Klemenz et al., 1987) which has the white gene, under the control of the hsp70 promoter, as the selectable marker. No transformants were recovered in those experiments. Experiments are in progress to find out the basis for this. Reports from other laboratories indicate that recovering transformants using the pW8 vector is difficult (J. Phillips, personal communication). Therefore, pUChsneo might be a better choice for these experiments.

To assess the effect of suppressor concentration on viability and suppression efficiency, it would be helpful to be able to control the expression of the suppressor tRNA genes. Since transfer RNAs are transcribed by RNA polymerase-III from internal promoters, this could be difficult. But recently, there have been reports of polymerase-III transcribed genes having polymerase-II like promoters (for example, in the case of U6 snRNA). It would be interesting to see if the expression of suppressor tRNA could be placed under the control of a heat shock promoter. If that could be done, germline transformations of the construct could be performed and transformants recovered at a temperature at which the suppressor tRNAs are expressed at the lowest level. Then, using those transformants, expression levels and the corresponding level of suppression could be studied at different temperatures. Such lines would also allow studies of the effects of different levels of suppressor tRNAs on the survival of the organism. This, of course, implies that the suppressor tRNA constructs placed under the control of the polymerase-II promoter would be insensitive to RNA polymerase III activity. Several control experiments could be done to test the feasibility of this line of experimentation. For example, a wild type tRNA gene under the control of a heat shock gene promoter could be over-expressed in the genome of Drosophila. This would be expected to be non-toxic, since the tRNA is not capable of causing read-through translation, a characteristic unique to suppressor tRNAs. A similar control experiment would express a mutant tRNA gene with linkers in the anticodon region. The expression of such a nonfunctional gene could be measured on the gels or Northern blots. Transcripts from such a gene would also be expected to be non-toxic.

Transcription levels could also be increased by bringing together transposons from different lines into a single strain by genetic crosses, thereby increasing the copy number per line. If each integrated suppressor tRNA gene was functional at a very low level (< 0.5%), then putting two or more in the same genome might result in a suppressor activity

that would be the sum of the individual suppressor tRNA activities. Then, it can be seen if this would result in any demonstrable level of suppression.

Studies of this nature would help us understand more of the phenomenon of tRNA-mediated suppression in Drosophila and might lead to the elucidation of the complex molecular mechanisms underlying this very fascinating field of informational suppression.

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